UNIVERSITY OF ALBERTA

Tellurite Resistance in *Escherichia coli*

BY

Yunfang Hou

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UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Tellurite Resistance in *Escherichia coli*** submitted by **YUNFANG HOU** in partial fulfillment of the requirements for the degree of Master of Science.

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Abstract

In this study the homology among all the cloned Tellurite (TeO_3^{2-}) resistance (Te^r) determinants and their incidence among plasmids within different incompatibility groups were determined. The IncPα Te^r determinant was found only within the Inc Pα plasmids. The IncHI2 (pMER610) and IncHI3 (MIP233) Te^r determinants were closely related and were present in IncHI2 and IncHI3 plasmids as well as in IncHII plasmids. This Te^r locus was identified within the *Xho*I "E" fragment in the IncHII plasmid pHH1508a. A small degree of homology was present between the R478 Te^r determinant and the Te^r determinants of pMER610 and MIP233. None of the Te^r determinants was homologous to the *ars* determinant in pUM3 (Mobley *et al.*, 1983), which was also found to confer a moderate level of Te^r (Turner *et al.*, 1992).

Earlier studies (Walter, 1990, Ph.D thesis) reported that the plasmid pDT1364 had been created by cloning the Te^r region of pHH1508a into the *Sal*I site of pUC8. However the origin of pDT1364 Te^r determinant from pHH1508a (Walter, Ph.D. thesis, 1990; Walter et al., 1991b) were shown to be incorrect in this study. The 6.8kb insert of pDT1364, including the *tehAB* genes, had originated from the *E. coli* K-12 chromosome instead of the IncHII plasmid pHH1508a. The *tehAB* genes were localized on the *E. coli* K-12 map at a position between kb1508 and kb1510 (32.3 min coordinate). They were found to be widely conserved in *E. coli*, but were not found in none of the other species tested.

Although the *tehAB* genes result in a high level of Te^r with a MIC of K_2TeO_3 128μg/ml in pDT1364 using pUC8 as a vector, they usually do not specify Te^r when present in the *E. coli* chromosome. Most of the *E. coli* strains had MICs of 2 to 8μg/ml. Only one strain, *E. coli* BB4 had a very high MIC over 256μg/ml. A few strains including JC1569,

JM105 and DH5α were very susceptible to K_2TeO_3 and had very low MICs at 0.4, 0.1 and 0.64μg/ml.

PK1439 and ECOR 17 were the only two *E. coli* strains which were found to be devoid of *tehAB* operon. The large deletion mutant PK1439, containing a 140-kb deletion within the terminus region, specified an MIC (0.2μg/ml) about 20 times lower than most other *E. coli* strains. However the strain ECOR 17 still specified an MIC of 2μg/ml which is very similar to the MICs of most *E. coli* strains carrying chromosomal *tehAB* genes. Te^r was fully complemented by pDT1364 in ECOR 17 but was not complemented by pDT1364 in PK1439. It is likely that other genes as yet unidentified are required for expression of Te^r by the *tehAB* genes.

The conservation of the *tehAB* genes in the majority of *E. coli* strains may indicate that the genes have important functions. Other possible functions of the *tehAB* genes need to be investigated.

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1. INTRODUCTION

1.1 Physical and Chemical Properties of Tellurium

Tellurium (Te), discovered by Muller von Reichenstein in 1782, was identified as a distinct element and was named by Klaproth in 1798 (Steinberg *et al.*, 1942). Derived from the Latin *tellus*, or earth, it is an element of Group VIA of the Periodic Table, which includes oxygen, sulfur, selenium and polonium. The atomic number of Te is 52 and atomic weight is 127.6. Te has a specific gravity of 6.26, melts at 452.1 ^{o}C and boils at 989.8 ^{o}C. It is chemically similar to sulfur and selenium, but it is more electropositive, more basic and more metallic (Hampel, 1961). Tellurium resembles a metal in its physical properties. Elemental tellurium, which is insoluble in water (Cooper, 1971), is a silver-white metalloid with a metallic lustre. It is a poor conductor of heat and electricity; its conductivity does not, like that of selenium, vary with the amount of light falling on it. Like sulfur and selenium, it has a number of oxidation states in even numbers -2, +2, +4 and +6. It forms salts of tellurous and telluric acid with many metals. The principal soluble salts are those of sodium and potassium, with which it also forms tellurides by direct union.

1.2 Production of Tellurium and Its Presence in the Environment

1.2.1 Production

There are no ore deposits which can be mined for tellurium alone. The recovery of tellurium is linked with the production of copper, silver, lead and gold. Accumulation in the anodic sludge during electrolytic refining of copper is the main source of tellurium production, accounting for 80% of the world's supply of tellurium. The remainder is recovered from slimes and slags of lead refining, and from pyrite and pyrrhotite burned in pulp and paper mills and sulfuric acid plants.

1.2.2 Environmental Distribution

Tellurium is one of the rarest elements on earth. It is estimated to rank seventy-fifth in abundance of the elements on the earth's crust, 0.002ppm (Hampel, 1961; Mason, 1958). However, tellurium is widely distributed. It has been found to be in certain coals (Goldschmidt, 1958), which suggests that plants may have absorbed the element from soil in ancient times. It is also present in some seleniferous soils (Beath *et al.*, 1935). According to Schroeder *et al.* (1967), who have made an extensive survey of tellurium in foods, waters, vegetation, animal tissues and human organs, blood and urine, tellurium is found in two-thirds of foods, vegetables and humus. Interestingly fresh garlic contains a relatively large amount of tellurium, suggesting (Schroeder *et al.*, 1967) that this plant accumulates tellurium from soil. In dried garlic, however tellurium could not be detected. Tellurium contained in garlic is dimethyl telluride, a volatile compound, which gives garlic its characteristic odor and taste (Schroeder *et al.*, 1967). Tellurium concentrations in water are very low as no tellurium could be detected in 20 samples of tap water from one location in the U.S.A. (Schroeder *et al.*, 1967). No spectrographically detectable quantities of tellurium were found in the principal U.S.A. rivers (Durum, 1960). In man there appears to be some 600 mg of tellurium, with the largest amounts in bone. This level exceeds those of all other trace elements but iron, zinc and rubium (Schroeder *et al.*, 1967). Daily oral human intake is about 100 μg (Nason and Schroeder, 1967). Urinary excretion approximates the intake. Tellurium is also found in both serum and red blood cells (Schroeder *et al.*, 1967). However, there appears to be no nutrient or biological role for tellurium. Tellurium is present in soils in large enough amounts to be absorbed by plants. Because tissues of herbivores also contain tellurium, a tellurium cycle, from soils to plants to animals, probably occurs in nature, accounting for part of the exposures of human beings. Most human exposure probably results from industrial contamination.

1.3 Uses of Tellurium

1.3.1 Industrial Applications

Tellurium is used widely in various industrial processes. In the rubber industry, it acts as a secondary vulcanizing agent to increase physical, thermal and chemical resistance of rubber and as an anti-aging agent in the final product; In metallurgy, it is used as an additive to iron, steel and copper to improve machinability and surface resistance to corrosion and stress; In the chemical industry, it works as a catalyst; In the microelectronic industry, it is used as a semiconducting compound for thermoelectric applications. It has been used as a coloring agent in chinaware, porcelains, enamels and glass as well as as a reagent in producing black finish on silverware. Tellurium is also used in nuclear reactors to produce radioactive iodine (I^{131}).

1.3.2 Therapeutic Uses

Tellurium has been of some medical interest. It has been used to treat syphilis (Fournier and Levaditi, 1926) and leprosy (Stanziale, 1929), and to reduce the sweating of patients suffering from phthisis (i.e. tuberculosis) (Neusser, 1890). More recently, tellurite has been suggested as a potential anti-sickling agent due to its ability to increase mean cell volume and decrease mean corpuscular haemoglobin concentration (Asakura *et al.*, 1984). A new and novel application has been proposed for tellurium as an immunomodulating drug, referred to as AS-101 (Sredni *et al.*, 1987, 1988a, 1988b), which is a low molecular weight organic tellurate compound, ammonium trichloro (dioxoethylene-O, O'-) tellurate. This compound is found to have immunomodulating properties without any significant toxic side-effects in rats. It could be a potential drug for AIDS and cancer patients.

1.3.3 Uses of Tellurium Compounds in Microbiology

Differential media are used to isolate specific microorganisms from contaminated material. Certain pathogenic species are naturally resistant to tellurite. This resistance forms the basis of certain diagnostic techniques in clinical microbiology. Tellurite has been used in a variety of selective bacteriological media and differential tests.

As early as 1900, potassium tellurite was introduced into bacteriology by Klett (Klett, 1900). He observed that many microorganisms, including the diphtheria bacillus could reduce tellurite. In 1912, Conradi and Troch proposed a medium containing tellurite as an aid in the diagnosis of *Corynebacterium diphtheriae*. On this medium the colonies of the organism have a characteristic black color due to the reduced tellurium and the medium exerts a selective bacteriostatic effect, allowing the diphtheria bacillus to grow but inhibiting most other microorganisms found in the throat. Thus for many years, potassium tellurite has been used as a constituent of selective media for the isolation of diphtheria bacillus (MacFaddin, 1985; Saragea *et al.*, 1979).

During isolation of *Corynebacterium diphtheriae* using tellurite-containing media, it was observed that some *Staphylococci* were also capable of reducing tellurite and had the same colony morphology. Thus black colonies on a tellurite containing medium must always be examined by Gram-stained smear (Hoeprich *et. al.*, 1960). In 1949 Ludlam first suggested that tellurite be used as a selective agent for isolation of pathogenic *Staphylococci*. Since then tellurite reduction has been recommended for laboratory determination of potential pathogenicity of *Staphylococcus* species isolated from clinical sources (Hoeprich *et. al.*, 1960).

Besides *C. diphtheriae* and certain *S. aureus*, group D *Streptococcus* (the *enterococcus*) is another important bacterium which is naturally resistant to tellurite

(Skadhauge, 1950; Appleman and Heinmiller, 1961). Potassium tellurite as a selective agent for the isolation of *Listeria monocytogenes* was first recommended in 1944 by Schoer, who recommended the use of potassium tellurite as an inhibitor of gram-negative organisms. This finding was confirmed (Gray *et al.*, 1950), in spite of reports that potassium tellurite inhibited the growth of some strains of *L. monocytogenes* (Leighton, 1979). Potassium tellurite is still incorporated into different isolation media for *Listeria* spp, usually associated with other selective agents (Blanco *et al.*, 1989; Buchanan, 1990; Khan *et al.*, 1973). Since potassium tellurite is reduced by *Listeria* spp., it is also used as a differential agent (Buchanan *et al.*, 1987). Recently, it was found that potassium tellurite enhances the hemolytic phenotype of *L. monocytogenes* (Fernandez-Garayzabal *et al.*, 1992), probably by the enhancement of the cytolytic effect of listeriolysin O (LLO).

Mycobacteria also produce black colonies on culture media containing tellurite. This property has long been used as a rapid test for the viability of tubercle bacilli (Corper, 1915; Kilburn *et al.*, 1969). In addition, the resistance of *Alcaligenes* to tellurite is used to differentiate *A. faecalis* and *A. denitrificans* from *Bordetella bronchiseptica* (Johnson and Sneath, 1973). In summary, potassium tellurite has been used extensively as a selective and differential agent in clinical microbiology.

1.4 Toxicity of Tellurium in Animals and Humans

The toxicity of tellurium varies with its state of oxidation. Tellurium toxicity increases when proceeding from the element to tellurate (TeO_4^{2-}), tellurite (TeO_3^{2-}) and telluride, hydrogen telluride (H_2Te) being the most toxic compound (Scansetti, 1992). Elemental tellurium is the least poisonous form of tellurium and has little toxicity (Cerwenka et al., 1961; De Meio, 1946). Tellurite is more toxic than tellurate, regardless

of the route of administration. The toxicity of tellurite is ten times the toxicity of tellurate when given by intraperitoneal injection of rats (Franke, 1937), but only twice as toxic when fed in chronic studies (Fishbein, 1977). Oral toxicity is somewhat lower than parenteral, presumably because of reduction to metallic tellurium in the gastrointestinal tract.

1.4.1 Toxicity in Animals

Early observation on the toxic effects of tellurium in animals emphasized two phenomena: the garlic odor of the breath and tissues, and a deep bluish-black coloration (Browning, 1969). Apart from these, digestive disturbance, lack of growth, emaciation, somnolence and loss of hair are also commonly observed. Severe poisoning can result in death, which is mainly due to respiratory paralysis. Organs most affected in tellurium poisoning of animals are liver, kidney, nervous system, lungs and the gastrointestinal tract. The effects of tellurium on the nervous system of rats include paralysis of hind legs, alteration of conditioned reflexes and reduced learning ability (Glover and Vouk, 1979).

1.4.2 Toxicity in Humans

Although in animals tellurium compounds have proved lethal in high dosage, tellurium has not been found to be a very hazardous element in the industrial sense. Reports of acute occupational tellurium intoxications are rare. The main symptoms and signs of tellurium poisoning in humans include a strong and lasting garlic odor in the breath, sweat and urine, a metallic taste and dryness in the mouth, anorexia, nausea, headaches, loss of appetite, somnolence, and suppression of sweat. An unusual feature is the bluish-black discoloration of the webs of the fingers and streaks on the face and neck (Fishbein, 1977; Muller *et al.*, 1989).

Fatal cases due to occupational tellurium exposure have not been described to date (International Labour Office, 1983). The only lethal tellurium intoxications reported thus far occurred after accidental injection of sodium tellurite in place of sodium iodide in retrograde pyelography (Keall *et al.*, 1946).

1.4.3 Carcinogenicity

Schroeder and Mitchener (1971, 1972) exposed mice for their life time to sodium tellurite and sodium tellurate in drinking water at 2 ppm tellurium. Neither hexavalent nor tetravalent forms of Te had carcinogenic activity. However, a significantly increased incidence of chromosome breakage was observed in human leukocytes treated *in vitro* with sodium tellurite (1.2×10^{-8} M) and ammonium tellurite (2.4×10^{-7}M) (Patton and Allison, 1972).

1.5 General Considerations of Tellurite Resistance (Te^r)

Tellurite (TeO_3^{2-}) is toxic to most microorganisms, particularly gram-negative bacteria (Fleming, 1932; Scala and William, 1963). Some gram-positive bacteria are naturally resistant to tellurite, including *Corynebacterium diphtheriae* (Conradi and Troch, 1912), *Streptococcus faecalis* (now called *Enterococcus faecalis*) (Skadhauge, 1950; Appleman and Heinmiller, 1961) and most strains of *Staphylococcus aureus* (Hoeprich *et. al.*, 1960). Gram-negative bacteria are usually sensitive to tellurite, but tellurite-resistant strains can be isolated frequently from hospital and urban sewage, as well as film reprocessing sludge (Taylor and Summers, 1979). The resistance in gram-negative bacteria is usually plasmid-mediated and is often linked with other phenotypes, including resistance to bacteriophages, colicins and to multiple antibiotics. Thus tellurite resistance (Te^r) by plasmids may confer a selective advantage on bacteria.

Bacterial plasmids that share either replication control or partitioning functions compete for stable inheritance, are termed incompatible, and are placed in the same incompatibility (Inc) group. Plasmids of several incompatibility groups have been shown to mediate resistance to tellurite. In the *Enterobacteriaceae*, most of the tellurite-resistant plasmids belong to the IncHI, IncHII and IncP groups (Taylor and Summers, 1979; Taylor and Levine, 1980; Bradley *et al.*, 1982; Bradley, 1985). A few of them have not been classified (Taylor and Summers, 1979; Hughes and Datta, 1983).

1.6 Tellurite Resistance Encoded by Incompatibility H Group Plasmids

1.6.1 Incompatibility H Group Plasmids

An IncH plasmid was first isolated from a strain of chloramphenicol-resistant *Salmonella typhi*, which was responsible for the first Mexican epidemic of chloramphenicol-resistant typhoid fever in 1972. The plasmid was shown to be responsible for resistance to chloramphenicol and to several other antibiotics. It was classified into a new incompatibility group designated "H" (Anderson and Smith, 1972). IncH plasmids have since been shown to encode multiple drug and metal resistances in members of the family *Enterobacteriaceae* (Anderson, 1975; Summers and Jacoby, 1977; Taylor and Levine, 1980). Plasmids of the H group are all very large (greater than 100 megadaltons), a characteristic that has made them rather more difficult to analyze physically and genetically than plasmids of other groups.

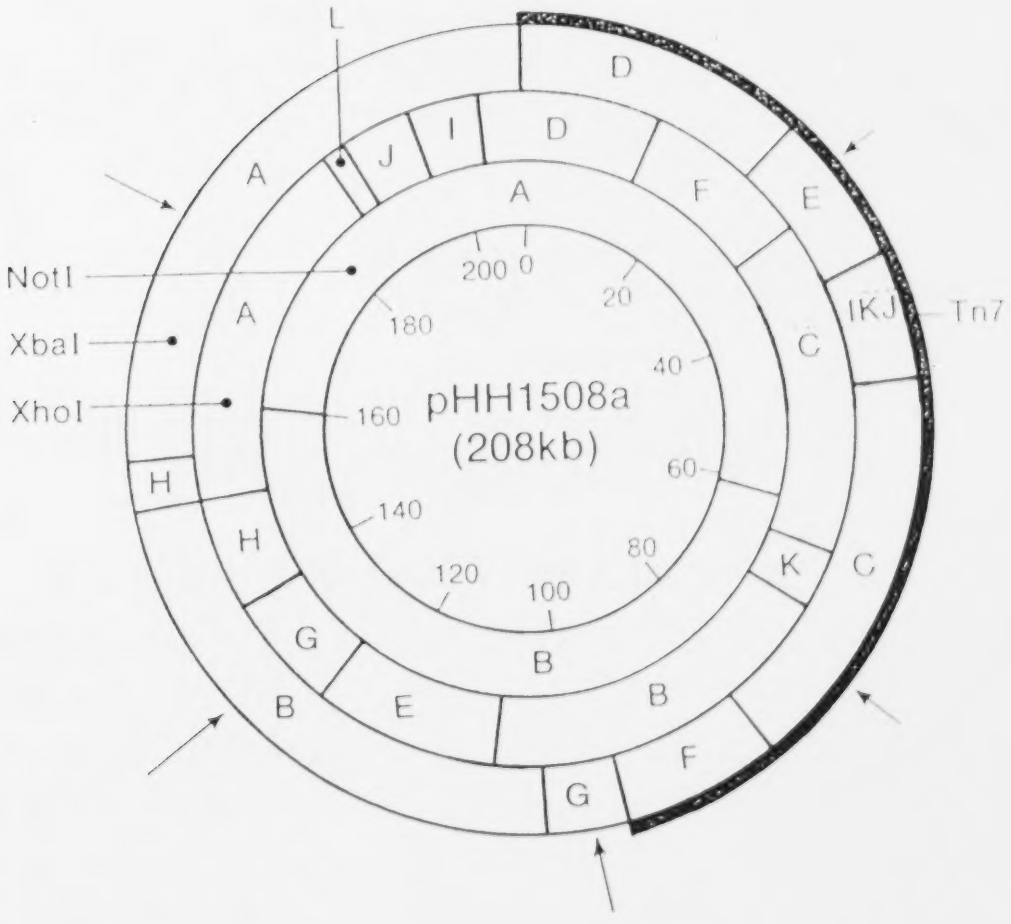
Two major IncH groups, IncHI and IncHII, are now recognized (for reviews of H plasmids, see references Maher and Colleran, 1987; Taylor, 1989). IncHI plasmids are thermosensitive for conjugation, i.e. transferring optimally at 26 to 30 ^{o}C and negligibly ($< 10^{-8}$) at 37 ^{o}C (Rodriguez-Lemoine *et al.*, 1975; Taylor and Levine, 1980). IncHII plasmids differ from IncHI plasmids in that they are non-thermosensitive for transfer, are

not repressed for pilus synthesis, and transfer at high frequencies (Bradley *et al.*, 1982). On the basis of DNA-DNA hybridization studies, the IncHI group of plasmids is further divided into three subgroups, IncHI1, IncHI2 and IncHI3 (Whiteley and Taylor, 1983). There are many members in the IncHI1 and IncHI2 subgroups, but there is only one, MIP233, in the IncHI3 subgroup (Roussel and Chabbart, 1979). All IncH plasmids specify thick, flexible pili (Bradley, 1980), except for IncHI3 plasmid MIP233, which specifies rigid pili (Bradley, 1986).

Restriction endonuclease maps have been generated for some IncH prototype plasmids, including the IncHII prototype plasmid, pHH1508a (208 kb) (Yan and Taylor, 1987; Figure 1.), the IncHI1 prototype plasmid, R27 (182 kb) (Newnham and Taylor 1990; Taylor and Brose, 1985; Taylor *et al.*, 1985) and the IncHI2 prototype plasmid R478 (272 kb) (Whelan and Colleran, 1992). They are all valuable to the molecular studies of IncH plasmids.

Te^r is commonly mediated by IncHI2, IncHI3 and IncHII plasmids, but has not yet been shown to be encoded by any plasmids of the IncHI1 subgroup. A 9.3 kb *Hin*dIII fragment containing the Te^r determinant of the IncHII plasmid pHH1457b has been cloned into pUC13 digested with *Hin*dIII and the clone was named as pDAK2 (D. Keane, M.Sc. thesis, 1990). Two other clones, MB7H5 (Whelan and Taylor, unpublished data) and pKFW6b which contained the Te^r determinants from MIP233 and R478, respectively have also been constructed (Whelan, Ph.D. thesis. 1992. see Table 3). These three Te^r determinants have not been sequenced. The homology among them and other Te^r determinants is yet to be determined. The Te^r determinant of the IncHII plasmid pHH1508a was reported to consist of *tehA* and *tehB* genes and be contained in pDT1364 (Walter, Ph.D. thesis, 1990). It has already been sequenced. The Te^r

FIG. 1. Restriction map of pHH1508a. Restriction endonuclease sites on the plasmid pHH1508a for *Xba*I, *Xho*I and *Not*I are shown in the outer circles. Map positions in the plasmid are shown in the center in kilobases. Restriction fragments are labelled alphabetically in order of decreasing size. Regions of the plasmid which are required for conjugative transfer are indicated by arrows. (modified from Yan and Taylor, 1987 by Walter, 1990)



determinant from the IncHI2 plasmid pMER610 (Jobling and Ritchie, 1987) has also been well characterized.

1.6.2 The Te^r Determinant of the IncHI2 Plasmid pMER610

The plasmid pMER610 is a large (>250 kb) conjugative plasmid belonging to the IncHI2 subgroup. It was originally identified from a gram-negative mercury-resistant bacterium, *Alcaligenes* isolated from the River Mersey. Then it was transferred by conjugation to *Escherichia coli* AB1157. In addition to becoming mercury resistant as a consequence of the acquisition of pMER610, this AB1157 derivative also acquired increased resistance to both tellurite and tellurate by more than 100-fold and expressed the phage inhibition phenotype (Phi).

The Te^r determinant of pMER610 has been cloned on a 3.55-kb DNA fragment. Both the original Te^r plasmid pMER610 and its subclone pMJ606 (which is numbered in our stock as pDT1798) encode inducible Te^r by prior exposure to tellurite at levels (1μM) sub-toxic to the sensitive AB1157 (Jobling and Ritchie, 1987). Nucleotide sequence analysis has identified five ORFs, which are designated *terA-terE*, respectively (Jobling and Ritchie, 1988; Hill *et al.*, 1993). The sizes of their gene products predicted from the DNA sequence are 37, 14, 38, 20 and 20 kilodaltons (kDa), respectively. Four polypeptides of equivalent sizes have been expressed in "maxi-cells" carrying the *terA*, *B*, *D* and *E* genes (Jobling and Ritchie, 1987). Only polypeptide TerC, which has a predicted sequence of 346 amino acids, has not been detected in maxi-cells. Hydrophobicity profiles predict that the TerC polypeptide is an inner membrane protein. This may explain why this polypeptide could not be found using the maxi-cell procedure. Although TerC has not been observed in maxi-cells, the gene has been shown by gene fusions to be both transcribed and translated (Hill *et al.*, 1993).

Analysis using Tn*1000* insertion mutagenesis indicated that terA and terB may be regulatory genes, whereas *terC, D* and *E* are the structural genes determining Te^r. The genes, *terD* and *E*, appear to encode the same function. The nucleotide and predicted amino acid sequences of *terD* and *terE* show considerable homology, 66.5% and 65.2% respectively. It suggests that the two genes may have arisen from a gene duplication event (Jobling and Ritchie, 1988). Although partial activity is retained when *terE* is deleted, expression of both *terD* and *terE* is required for full resistance (Jobling and Ritchie, 1988).

1.7 Tellurite Resistance Encoded by IncP Plasmids

1.7.1 Plasmids of the P Incompatibility Group

IncP plasmids are promiscuous, i. e. they are capable of conjugal transfer among, and stable maintainance in, almost all gram-negative bacterial species (for reviews of P plasmids see Thomas and Smith, 1987; Schmidhauser *et al.*, 1987; Thomas, 1981). Such promiscuity is of interest as it relates to the spread of antibiotic resistance and its application to genetic manipulation in diverse bacterial species.

The P plasmids have been divided into two subgroups, IncPα and IncPβ, based on DNA homology studies of their transfer origins (the *oriT*s) (Yakobson and Guiney, 1983). By using Southern blotting to compare the *Hae*II fragments carrying homology to the *oriT* region of RK2, only two patterns of hybridization were observed. Most of the plasmids showed a single band of the same size as that from RK2 and were designated IncPα, whereas R751, R906, and R772 showed two bands of smaller size and lower homology and were designated IncPβ. Studies using probes derived from the *trf*A and *ori*V regions of RK2 (Chikami *et al.*, 1985) and the *pri* locus of RP4 (Lanka *et al.*, 1985) confirmed the validity of this subgrouping, and were supported by both heteroduplex

analysis (Sakanyan *et al.*, 1983; Villarroel *et al.*, 1983) and by complementation studies (Yakobson and Guiney, 1983). The IncPα plasmids are generally larger than the IncPβ plasmids and encode unexpressed resistance to tellurite which can be selected by growth on medium containing tellurite. In contrast, IncPβ plasmids do not express Te^r (Bradley, 1985).

1.7.2 The Te^r Determinant of the IncPα Plasmid, RK2

The 60-kb plasmid RK2 was originally isolated in 1969 from an outbreak of R-factor-mediated carbenicillin resistance in *Pseudomonas aeruginosa* in burn patients in Birmingham, England (Holloway and Richmond, 1973; Ingram *et al.*, 1973). It belongs to the Pα incompatibility group. This plasmid has been studied extensively because it can replicate and be stably maintained in a wide variety of gram-negative bacteria and also carries resistance to ampicillin, kanamycin, and tetracycline as well as several transposable elements (Thomas and Smith, 1987). RK2 and plasmids named RP4, RP1, R18 and R68, which were isolated from different patients have been found to be indistinguishable by restriction endonuclease mapping and by heteroduplex analysis and thus their names have been used interchangeably (Burkardt *et al.*, 1979; Currier and Morgan, 1981).

By using Tn*7* insertion mutagenesis, the cryptic RK2 determinant for Te^r was located at coordinates 56-0-1.5 kb on the RK2 map between the *korA* and the *kilA* genes, which are involved in plasmid replication control (Taylor and Bradley, 1987). The *kilA* gene, identified by Figurski *et al.* (1982), was named for the lethal effect that was observed when it was cloned separately from the gene *korA*. The gene *korA* named for *k*ill-*o*ver*r*ide, negatively regulates transcription from the *kilA* promoter (Young *et al.*, 1985, 1987), and prevents the lethal effects of the *kil* genes on the host (Figurski *et al.*, 1982).

The Te^r determinant of RK2 has been cloned into pACYC184 and pUC8 respectively, creating plasmids pDT1555 and pDT1558 (Walter *et al.*, 1991a). Transcriptional and translational fusions were constructed within the *kilA* and Te^r genes carried on pDT1555 using the transpositional phage mini-mu. These fusions indicated that the Te^r genes were transcribed in the same direction as *kilA* and that transcription and translation of the cloned *kilA* gene was occurring and may not be lethal to the bacterial cell even in the absence of *korA*. The nucleotide sequence of this region was determined. The operon contains three ORFs, *kilA*, *telA* and *telB* (Walter and Taylor, 1989; Walter *et al.*, 1991a), all of which are necessary for expression of high-level-resistance to tellurite (Walter, 1990; Walter *et al.*, 1991a; Goncharoff *et al.*, 1991). The first ORF, *kilA*, codes for KilA, a 28-KDa hydrophilic polypeptide that is probably located in the cytoplasm. The second ORF, *telA*, codes for a 42-KDa hydrophilic polypeptide which is likely to be located in the cytoplasm. The third, *telB*, codes for a 32-KDa hydrophobic polypeptide, which appears to be located in the inner membrane of the bacterial cell, since fusions of TelB to alkaline phosphatase were obtained by using Tn*phoA* (Walter *et al.*, 1991a). All three polypeptides were detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) after overproduction using the T7 RNA polymerase/promoter system. The same three proteins were produced when Te^s and Te^r derivatives of RP4 were expressed in an *in vitro* transcription-translation system (Walter *et al.*, 1991a).

Although no difference could be detected between the restriction maps of $RK2Te^r$ and $RK2Te^s$, a difference between DNA sequences of the Te^r and Te^s derivatives was observed. There is a single Ser (Te^s) to Cys (Te^r) missense mutation at amino acid residue 125 in *telB*, which appears to be responsible for the phenotype change from Te^s to Te^r (Walter *et al.*, 1991a).

Interestingly, there is another Cys residue found nearby in *telB* at amino acid residue 132. Since two closely spaced Cys residues are found in a number of proteins involved in metal binding such as mercuric reductase (MerA), periplasmic mercury-binding protein (MerP), the cadmium resistance protein (CadA) (Nucifora *et al.*, 1989) and glutathionine reductase (Perham, 1987), Walter and Taylor (1992) proposed that the two cysteine residues (Cys-125 and Cys-132) in Tel B may be located on the cytoplasmic face of the protein where they could potentially be involved in binding to ligands.

1.8 Tellurite Resistance Determinant Encoded by pDT1364

Plasmid pDT1364 consists of pUC8 with a 6.8kb insert believed to originate from the large IncHII plasmid pHH1508a (208kb) (Walter, 1990, Ph.D thesis). However, the cloned fragment did not consistently hybridize to pHH1508a plasmid DNA, although this was considered to be the source of the clone. In addition, *E. coli* K-12 J53-1 (pDT1364) consistently had a much lower MIC of tellurite (128 μg/ml) than *E. coli* K-12 J53-1 (pHH1508a) (1024 μg/ml). Further studies were thus required to verify the origin of this clone.

A 1.8-kb DNA fragment of pDT1364 was shown to be required for tellurite resistance by subcloning and Tn*1000* mutagenesis. The DNA sequence of this region was determined (Walter *et al.*, 1991b). No homology was detected between this Te^r determinant and either pMER610 or RK2 Te^r (Walter and Taylor, 1992). No similar sequence was present in either of the following protein data bases, SWISS-PROT, PIR and KeyBank™. The fragment contains an operon consisting of two genes which have been named *tehA* and *tehB* (Walter *et al.*, 1991b). The *tehB* gene encodes a 23-kDa polypeptide which is relatively hydrophilic and is probably located in the cytoplasm. The

tehA gene encodes a 36-kDa polypeptide which migrates with an apparent molecular mass of 28 kDa in SDS-PAGE due to its high hydrophobicity. Five to ten membrane-spanning domains have been identified and the polypeptide is likely to be located in the inner membrane of the bacterium. Three cysteine residues appear to be located within 3 central putative membrane-spanning domains and these residues may play a role in resistance by binding to ligands (Walter and Taylor, 1992).

1.9 Tellurite Resistance Mechanism(s)

Although the gene products of the above Te^r determinants are known, the mechanism(s) of Te^r is, as yet, not well understood. One common feature manifested by all tellurite-resistant cells is their black appearance when grown in the presence of tellurite. The true identity and nature of the black precipitate has been determined. As early as 1941, Morton and Anderson observed the black precipitate inside cells of *Corynebacterium diphtheriae* after growth on chocolate tellurite agar. They observed that the black precipitate was soluble in bromine water, an oxidizing agent, and that the precipitate was of high density in electron micrographs. It appeared highly probable that the black color was due to existence of tellurium metal. About 20 years later, Tucker *et al.* (1962), using X-ray diffraction analysis of whole cells, showed that the black precipitate produced in the presence of potassium tellurite consisted of metallic tellurium. Using the technique of electron spectroscopic imaging, Taylor *et al.* (1988) verified the earlier observations that the black intracellular deposits consisted of reduced metallic tellurium. Thus, the reduction of the soluble and toxic oxy-anions of tellurium to insoluble and non-toxic elemental tellurium has been suggested as a highly possible mechanism of bacterial resistance to tellurite.

Teral *et al.* (1958) reported that tellurite reduction occurs in *Mycobacterium avium*. They demonstrated that a protein fraction could reduce this salt in the presence of reduced nicotinamide adenine dinucleotide or malate and malic dehydrogenase and referred to this tellurite-reducing enzyme as tellurite reductase. A similar tellurite-reducing activity was found in cell-free extracts of *S. faecalis* (Thomas *et al.*, 1963).

Thermus spp., extremely thermophilic gram-negative rods, were found to be resistant to tellurite (Chiong *et al.* 1988a). Cell-free extracts of *Thermus thermophilus* HB8 and *T. flavus* AT-62 also catalyzed the *in vitro*, NADH-dependent reduction of potassium tellurite. Three different protein fractions with tellurite-reducing activities were identified (Chiong *et al.*, 1988b). Two exhibited high molecular weight and were composed of at least two different polypeptides. The protein in the third fraction was purified to homogeneity and had a single polypeptide of 53 to 54 kDa. The enzymes were also able to catalyze the reduction of sodium selenite and sodium sulfite *in vitro*. Chiong *et al.* (1988b) suggested that Te^r in *Thermus* spp. is due to the presence of these tellurite reductases. This Te^r did not appear to be plasmid-mediated and it has not been fully characterized yet.

The Te^r determinants from the IncHI2 plasmid pMER610, the IncPα plasmid RK2 and the plasmid pDT1364 have been cloned, sequenced and expressed. No apparent homology was seen at either DNA level or amino acid level among these three Te^r determinants (Jobling and Ritchie, 1988; Walter *et al.*, 1991c; Walter and Taylor 1989; Walter *et al.*, 1991a). Thus it is possible that different mechanisms are involved in Te^r mediated by these three determinants.

Since tellurite and selenite are chemically similar to sulfate, Scala and Williams (1963) proposed that tellurite and selenite could be reduced and thus detoxified by the

sulfate reduction pathway. The supporting evidence was that the sensitivity of *E. coli* bacterial cells to tellurite was enhanced by the presence of L-methionine. The presence of this exogenous reduced sulfur source would repress this pathway, thus decreasing the rate of detoxification of selenite and tellurite, and therefore increasing sensitivity to these anions. Recently this proposal was tested by expressing cysIJG, the genes encoding sulfite reductase and the Te^r determinant of pDT1364 together in a single host. However, no increase of tellurite resistance by adding sulfite reductase was found (Turner *et al.*, in preparation II). A new hypothesis was formulated that cysteine, NADH/NADPH and the electron transport system might be utilized as cofactors for the plasmid-encoded tellurite resistance. The decreased resistance to tellurite by the exogeneous reduced sulfur i.e. L-methionine might be due to inhibition of synthesis of cysteine which also shares the sulfate reduction pathway. This hypothesis requires additional testing.

Chromosomal Te^r has been found to result from reduced uptake of the toxic metal. Most isolates of *E. coli* are highly sensitive to tellurite, having a MIC of 0.25 to 1 μg/ml (Tomas and Kay, 1986; Taylor *et al.*, 1988). Spontaneous mutants of *E. coli* resistant to low levels of tellurite (~10 μg/ml) as well as to arsenate were obtained. These mutants were found to be defective in phosphate transport and were unable to grow on media containing low levels of phosphate (Tomas and Kay, 1986). Transport of phosphate was competitively inhibited by tellurite. Susceptibility to tellurite could be restored by a plasmid carrying the *phoB* region, which is involved in phosphate regulation. These results indicate that *E. coli* takes up tellurite by a phosphate transport system and that reduced uptake results in a low level of resistance (Tomas and Kay, 1986).

Studies have shown that *E. coli* cells harboring pDT1364, which expresses Te^r, contain much more black metallic tellurium than those harboring the IncPα Te^r genes (on

plasmids RK2Te^r and pDT1558) (Taylor *et al.*, 1988). Furthermore when bacteria containing the plasmid pDT1558 (carrying the IncPα Te^r genes) or pDT1364 were grown in broth containing tellurite, the filter-sterilized supernatant from the bacteria carrying the Te^r determinant of pDT1364 were found to reduce the toxicity of tellurite by 128-fold whereas the supernatant from cells carrying IncPα Te^r determinant only reduced the toxicity of the tellurite by 50% (Walter, 1990). These observations suggested that the pDT1364 and IncPα plasmids perhaps encoded different mechanisms of Te^r. Recent results examining the specific uptake of tellurite in *E. coli* harboring different Te^r determinants from Inc Pα, HII, HI2 and HI3 show little difference in uptake rates as compared to the control (Turner *et al.*, In preparation I). The above data make it difficult to assign a specific mechanism to these resistant determinants at this time.

Another type of transformation of tellurite has also been observed with *P. aeruginosa* and *T. thermophilus* (Summers and Jacoby, 1977; Chiong *et al.*, 1988a). Both microorganisms, when growing on suitable permissive levels of potassium tellurite, produce a strong garlic odor. This odor indicates the formation of an alkylated, volatile form of tellurium, probably methyl or dimethyltellurium (Fleming and Alexander, 1972). In contrast, Summers and Jacoby (1977) reported that *E. coli* strains do not produce this odor. Further experiments are needed to determine if methylation is one of the mechanisms used by bacteria to detoxify tellurite.

1.10 *Escherichia coli* K-12 Genomic Map and the Terminus Region

1.10.1 *Escherichia coli* K-12 Genomic Map

The genome of *Escherichia coli* consists of a single supercoiled, circular DNA molecule 4,720 kb in length. The DNA sequence of its genome has not been completed

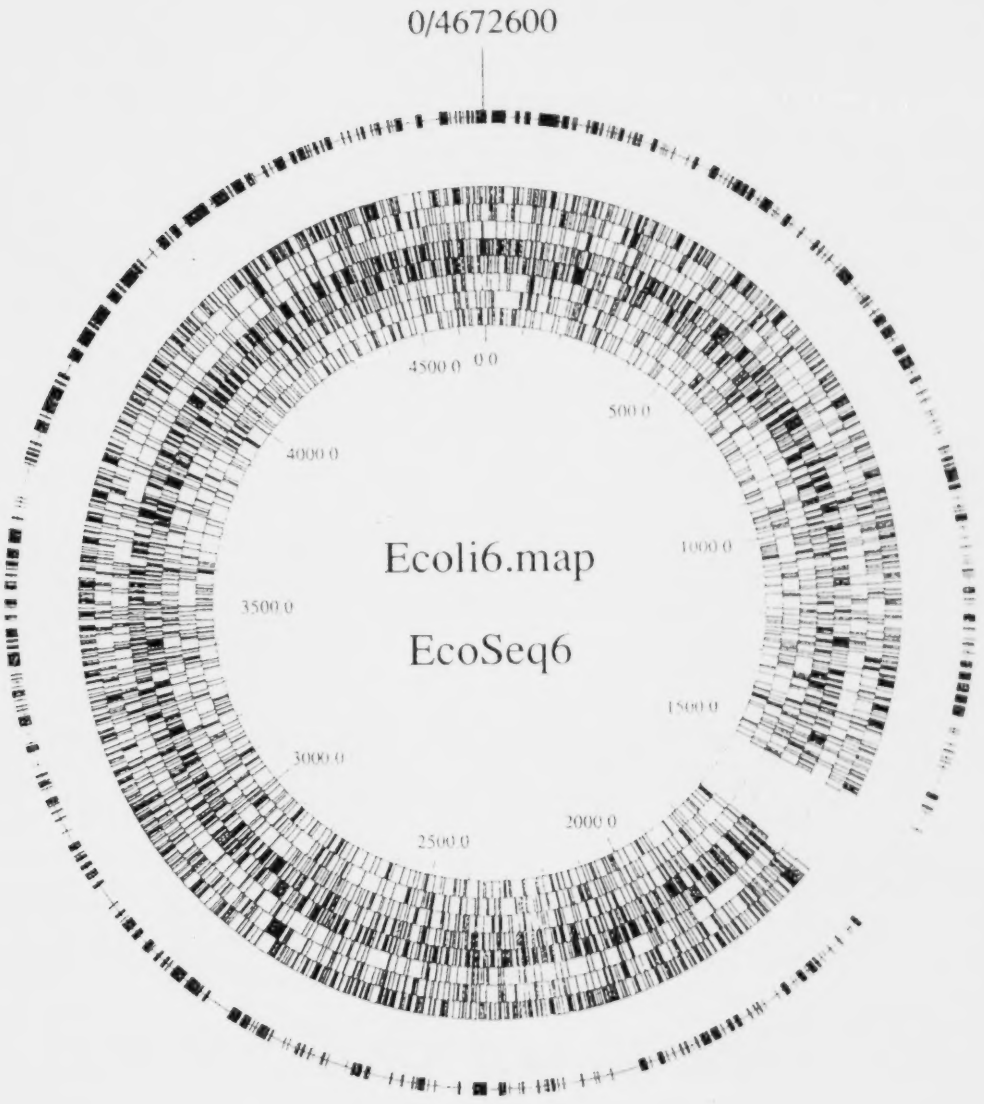


Figure 2. The sequenced areas within the circular chromosome of *Escherichia coli* K12. The eight inner circles represent the integrated genomic restriction map which is 4,672,600 bases in length and contains 7,882 restriction sites shown in bars. The outer circle indicates the sequenced areas. (Modified from Rudd, 1993)

yet. By July 1992, a total of 1,875,933 individual base pairs have been sequenced as a collective, uncoordinated effort (Rudd, 1993; Figure 2.). This corresponds to a total of 40% of the entire *E.coli* chromosome. It is roughly predicted that a complete *E. coli* sequence may be known by 1998, using a noncoordinate effort only (Kroger, *et al.*, 1992). In spite of incomplete DNA sequence, the entire *E. coli* genome has been mapped genetically and physically (Kohara *et al.*, 1987).

A genetic map has been constructed, in which a series of genes are identified by mutant phenotypes and are ordered using information from genetic crosses (Bachmann, 1983, 1990). Gene positions were first approximated by means of Hfr crosses, which can in some cases be accomplished rapidly (Low, 1973) and then localized more precisely by cotransduction of genetic markers using phage P1. The units of the map are minutes as determined by time of entry of markers in interrupted conjugation experiments (Bachmann, 1976). Genetic maps portray linkages that aid in strain construction and are used to catalog information about genes by chromosomal locus.

A physical map was constructed by Kohara *et al.* (1987) by isolating a subset of the 3,400 lambda phage clones containing segments of the *E. coli* chromosome. It is actually a restriction map for eight six-base-recognizing endonucleases, namely *Bam*HI, *Hind*III, *Eco*RI, *Eco*RV, *Bgl*I, *Kpn*I, *Pst*I and *Pvu*II. A total of 381 selected clones are found necessary to cover virtually the whole of the *E. coli* genome with minimal overlaps. Four hundred and seventy-six clones were selected from the original collection to cover the entire genome with ample overlapping. This new collection was called the "miniset" (Kohara, 1990). The miniset library is now also available in an ordered array supplied on a single sheet of hybridization membrane, the *E. coli* Gene Mapping Membrane containing plaque lifts of the Kohara miniset. Thus researchers can quickly obtain DNA fragments containing a desired gene or genes with ease, once its (their) rough location on

the *E. coli* genetic map is known. Furthermore, these genes can be quickly located by hybridization to the commercially available Kohara miniset hybridization filters.

Since the genes of *E. coli* chromosome in the Kohara library are cloned in lytic phage vectors, the cloned gene(s) cannot be examined directly with respect to its (their) capability of complementing a mutation. To overcome this, it will be necessary to correlate the individual lambda phage clones with other types of clone sets. Recently the correlation of the Kohara library to the Clarke-Carbon library has been made (Nishimura *et al.*, 1992). The Clark and Carbon collection (Clarke and Carbon, 1975; 1976) consists of 2,200 strains that harbor hybrid *E. coli* K-12-ColEI plasmids (pLC plasmids). Each hybrid DNA circle contains a random piece of *E. coli* K-12 chromosomal DNA that was mechanically sheared to an average size of 14 kb and was ligated to ColEI DNA by the AT-tailing method (Nishimura *et al.*, 1992). Thus 518 pLC plasmids were located on the physical map by plaque hybridization to the Kohara miniset clones. Further identification of the remaining pLC plasmids by plaque hybridization is in still progress (Nishimura *et al.*, 1992).

An integrated *E. coli* genomic map (Rudd *et. al.*, 1990, 1992) has been made by using the 1983 *E. coli* genetic map (Bachmann, 1983), the genomic restriction map published by Kohara *et al.* (1987) and DNA sequence database entries. Thus one can look up a particular genetic map position (in minutes) and find the corresponding physical genomic map coordinates (in kb). Such a map will be of general utility to *E. coli* researchers.

1.10.2 The Replication Terminus Region

Examination of the genetic map shows that known genes of *E. coli* are not evenly distributed (Bachmann, 1990). Several regions contain relatively few identified genes. The most gene-sparse region of the *E. coli* chromosome is the replication terminus region which is located between min 31 and min 35 on the genetic map (Moir *et al.*, 1992). It is directly oppposite the origin of replication on the circular chromosome. Bidirectional replication forks that initiate at the replication origin meet in the terminus region at the end of the replication cycle (Kuempel *et al.*, 1973). One striking property of the terminus is that both clockwise- and counterclockwise-traveling replication forks are inhibited as they proceed across it (Kuempel *et al.*, 1977; Louarn *et al.*, 1977; Bouch *et al.*, 1982).

The functions encoded in the terminus were studied by obtaining strains with deletions in this part of the chromosome (Henson and Kuempel, 1985). A restriction map has been constructed for the terminus region by Bouche (1982). This greatly facilitates characterization of deletions. Henson *et al.* (1984) isolated mutants (PK1434, PK1430, PK1433 and PK1427) in which various parts of the terminus region have been deleted. The strains harboring 60 kb deletions in this region had no readily apparent mutant phenotype and studies of this strain suggested that this large region of DNA is not essential for normal growth (Henson *et al.*, 1984). A strain containing a larger deletion, that removed the entire terminus region along with 7-minutes (340 kb) of DNA, had an abnormal phenotype (Henson and Kuempel, 1985). The most dramatic property of this strain was the absence of the sites that inhibit clockwise- and counterclockwise-traveling replication forks. It also grew slowly, produced many nonviable cells which were filamentous, and appeared to have an induced SOS system (Henson and Kuempel, 1985).

In order to determine whether the terminus region consists of protein-coding sequences, Moir *et al.* (1992) cloned 44 kb (1 min) of terminus region DNA (that surrounding *trg* at 31.4 min) and examined its ability to catalyze protein synthesis *in vitro* or in minicells. The 1-min segment within the terminus region was found to code for a substantial number of polypeptides, indicating that the region as a whole, although dispensable by deletion and lacking genetic markers, nevertheless encodes a substantial number of polypeptides and thus cannot be said to be gene sparse (Moir *et al.*, 1992). The functions of these genes remain to be discovered, but the terminus region remains distinctly different from the rest of the chromosome in that it encodes such a large number of apparently non-essential proteins and no essential ones.

1.11 Objectives of This Study

In spite of extensive genetic and some biochemical characterization of tellurite resistance, additional experiments are required to understand the nature of bacterial tellurite resistance. In this thesis I will focus on the identification of the origin of the pDT1364 Te^r determinant. Secondly, I will determine the incidence of the three different tellurite resistance determinants specified by pDT1364 (previously reported from IncHII pHH1508a but will be proved to be from the *E. coli* chromosome in this study), pMJ606 (from IncHI2 pMER610) and pDT1555 (from IncPα RK2) (Table 3.) among plasmids of different incompatibility groups. Thirdly, the levels of Te^r specified by a variety of wild type plasmids and cloned fragments will be tested.

2. Material and Methods

2.1 Media and Solutions

In most of the experiments, Brain Heart Infusion (BHI) (Oxoid) broth and agar were used for growth of bacterial strains. Plasmid derivatives were cultured using media containing appropriate antibiotics. All plates containing tellurite and antibiotics were made from BHI agar or Mueller Hinton (MH) agar (Oxoid). The final concentrations of the selective reagents employed in media are listed in Table 1. Media used for isolation of bacteriophage DNA as well as the stock solution of gel electrophoresis buffer were made up as shown in Appendix I.

Table 1. Selective Agents

Agent	Final concentration (μg/ml)
Ampicillin	100
Carbenicillin	500
Chloramphenicol	32
Kanamycin	8
Nalidixic acid	24
Potassium tellurite	50
Rifampicin	100
Streptomycin	200
Tetracycline	8
Trimethoprim	10

2.2 Bacterial Strains and Plasmids

All ars-containing recombinant plasmids including pUM3 (*arsABC*) (Mobley *et al.*, 1983), pAB100 (*arsRAB*) (B. P. Rosen 1991, Personal communication), pUM11ΔBC (*arsA*) (B. P. Rosen 1991, Personal communication) and pWSU2 (*arsC*) (Rosen *et al.*, 1988) were kindly provided by Dr. B. P. Rosen, Department of Biochemistry, Wayne State University. The other parental and the recombinant plasmids used in this study are listed in Tables 2 and 3, respectively. The ECOR collection, a set of 72 *E. coli* strains was generously provided by Dr. W. L. Albritton, Department of Medical Microbiology and Infectious Disease, University of Alberta. All the other bacterial strains used for hybridization and PCR amplication are listed in Tables 4 to 6. The *E. coli* gene mapping membrane containing a recombinant λ phage library originally established by Kohara *et al.* (1987) was purchased from Takara Biochemicals Inc., CA. U.S.A..

Table 2. Bacterial Plasmids Used in This Study

Strain DT No.	Plasmid	Inc group	Phenotype[a]	Original Host	Reference
286	R26	Pα	ApCmGmKmSmSuTcHgr	*Pseudomonas aeruginosa*	Thomas & Smith, 1987
287	RP1	Pα	ApKmTc	*Pseudomonas aeruginosa*	Thomas & Smith, 1987
292	RP638	Pα	ApKmTc	*Pseudomonas aeruginosa*	Jacoby, 1977
293	RP527	Pα	ApCmGmKmSmSuTcHgr	*Serratia marcescens*	Thomas & Smith, 1987
294	R1033	Pα	ApCmGmKmSmSuTcHgr	*Pseudomonas aeruginosa*	Thomas & Smith, 1987
295	Rm16b	Pα	AmKmTc	*Pseudomonas aeruginosa*	Thomas &Smith, 1987
298	R702	Pα	KmSmSuTc	*Pseudomonas mirabilis*	Thomas & Smith, 1987
561	R1033	Pα	ApCmGmKmSmSuTcHgr	*Pseudomonas aeruginosa*	Thomas & Smith, 1987
83	R931	P2	SmTcHgrTer	*Pseudomonas aeruginosa*	Jacoby, 1977
84	R130	P2	GmSmSuHgr Ter	*Pseudomonas aeruginosa*	Jacoby, 1977
2203	pMG6	P2	CmGmKmSmSuTmCrrHgrTer	*Pseudomonas aeruginosa*	Jacoby *et al.*, 1983
2204	pMG31	P2	GmSmSuTcHgrTer	*Pseudomonas* sp.	Jacoby *et al.*, 1983
2199	pMG53	P2	ApCmGmKmSmSuTmAsrCrrHgrTer	*Pseudomonas fluorescens*	Jacoby *et al.*, 1983
2200	pMG56	P2	GmKmSmSuTmHgrTer	*Pseudomonas aeruginosa*	Jacoby *et al.*, 1983

Table 2. continued.

Strain DT No.	Plasmid	Inc group	Phenotype[a]	Original Host	Reference
2205	pMG64	P2	CmSmSuHgrPmrTer	*Pseudomonas fluorescens*	Jacoby *et al.*, 1983
2207	CAM	P2	Ter	*Pseudomonas putida*	Jacoby *et al.*, 1983
975	pHH1508a	HII	SmSuTpTer	*Klebsiella aerogenes*	Bradley, 1982
993	pHH1532b-1	HII	ApCmGmSuTmHgrTer	*Klebsiella aerogenes*	Bradley, 1982
994	pHH1457b	HII	ApCmGmKmSmSuTcTpHgrTer	*Klebsiella aerogenes*	Bradley, 1982
997	pHH1457-1	HII	ApGmHgrTer	*Klebsiella aerogenes*	Bradley, 1982
1	R476b	HI2	SmSuTcAsrHgr	*Serratia marcescens*	Taylor &Levine,1980
2	R826	HI2	ApCmGmKmSmTcAsrHgrTerPmrPhi	*Serratia marcescens*	Taylor & Levine, 1980
3	R826-1	HI2	ApCmGmKmSmAsrHgrTerPmrPhi	*Serratia marcescens*	Taylor & Levine,1980
4	R828	HI2	ApCmGmKmSmTcAsrHgrTerPmrPhi	*Serratia marcescens*	Taylor &Levine,1980
64	pWR23	HI2	LacScrTerPhi	*Salmonella tennessee*	Taylor & Levine,1980
65	MIP235	HI2	LacCmSmSuTerPhi	*Salmonella oranienburg*	Taylor & Levine,1980
71	RAS 5007	HI2	HgrTerPhi	*Citrobacter freundii*	Taylor & Levine,1980
78	TP116	HI2	CmSmSuHgrTerPhi	*Salmonella typhi*	Taylor & Levine,1980
109	R478	HI2	CmKmTcAsrHgrTerPhi	*Serratia marcescens*	Taylor & Levine, 1980
135	pAS-251-2	HI2	CmKmSmTcTer	*Salmonella typhimurium*	Taylor & Summers, 1979

Table 2. continued.

Strain DT No.	Plasmid	Inc group	Phenotype[a]	Original Host	Reference
416	pJT1	HI2	LacCmKmSmSuHgrTer	*Salmonella typhimurium*	J. Timoney[b]
447	R1022	HI2	CmSmSuTpTerPhi	*Enterobacter cloacae*	Taylor & Levine,1980
2206	pMG223	HI2	CmSmTcTer	*Klebsiella pneumoniae*	Jacoby *et al.*,1988
2197	pMG224	HI2	CmGmKmSmSuTcTpHgrTer	*Klebsiella pneumoniae*	Rice *et al.*, 1990
2198	pMG225	HI2	CmGmKmSmSuTcTpHgrTer	*Klebsiella pneumoniae*	Rice *et al.*, 1990
63	MIP233	HI3	ScrTerPhi	*Salmonella ohio*	Taylor & Levine,1980
106	pRG1251	HI1	ApCmSmSpSuTc	*Salmonella typhi*	Taylor & Levine,1980
68	RAS5002	ND	KmSmTcHgrTer	*Klebsiella pneumoniae*	Taylor & Summers, 1979
69	RAS5003	ND	CmKmSmTcHgrTer	*Klebsiella pneumoniae*	Taylor & Summers, 1979
70	RAS5005	ND	Ter	*Klebsiella pneumoniae*	Taylor & Summers, 1979
72	RAS5009	ND	HgrPmrTer	*Citrobacter freundii*	Taylor & Summers, 1979
73	RAS5012	ND	HgrPmrTer	*Citrobacter freundii*	Taylor & Summers, 1979

[a] Ap, ampicillin; Cm, chloramphenicol; Crr, chromate; Gm, gentamicin; Km, kanamycin; Sm, streptomycin; Sp, spectinomycin; Su, sulphonamides; Tc, tetracycline; Tm, tobramycin; Tp, trimethoprim; Asr, sodium arsenate; Hgr, mercuric chloride; Pmr, phenylmercuric acetate; Ter, potassium tellurite; Phi, phage inhibition; Lac, lactose fermentation; Scr, sucrose fermentation. These plasmid-determined properties are from the original description in the relevant reference.
[b] Department of Veterinary Microbiology,New York State College of Veterinary Medicine, Cornell University

Table 3. Recombinant Plasmids Used as Hybridization Probes

Plasmid	*E. coli* Host	Vector	Insertion Site	Insert Gene	Insert Size (kb)	Origin of probe	Resistance Marker	Reference
pDT1364	JM83	pUC8	*Sal*I	ch.[a]	6.8	*E. coli* ch.[a]	ApTer	Walter and Taylor, 1989
pDT1555	JM83	pACYC184	*Bam*HI/*Hin*dII	pTe^r	3.1	RK2	CmTer	Walter *et al.*, 1991
pMJ606[b]	AB1157	pLV59	*Pst*I	HI2Te^r	5	pMER610	CmTer	Jobling & Ritchie, 1987
pUM3[c]	HB101	pBR322	*Hin*dIII	*ars*ABC	4.3	R773	ApAsrTer	Mobley *et al.*,. 1982
pKFW6b	DH1	pUC13	*Eco*RI/*Sal*I	HI2Te^r	6.2	R478	Ap	K. F. Whelan, 1992[d]
MB7H5	DH5α	pUC119	*Hin*dIII	HI3Te^r	6.3	MIP233	ApTer	unpublished data[e]

a ch.=chromosome. The insert of pDT1364, which was reported to be from pHH1508a, is proved in this study to be from the *E. coli* chromosome.
b The lab assigned number for plasmid pMJ606 is pDT1798.
c The lab assigned number for plasmid pUM3 is pDT2196.
d Ph.D. thesis, 1992. University College Galway, Ireland.
e Whelan and Taylor

Table 4. Different *Escherichia coli* Strains Used in This Study

Strain No.	Strain	Genotype	Reference
DT384	W3110	*gal* λ^-	Bachman, 1972
DT902	J53-1	*pro met*	Bachman,1972
DT121	JC1569	*leuB6 tonA2 supE44 gal-6 λhisG1 rfbD1 galP63 argG6 xyl-7 mlA2 metB1 recA1 rpsL104*	Bachman,1972
DT192	RG192	*Δara leu lac* Rif^r	Taylor and Grant, 1977
DT1078	JM105	*thi rpsL endA sbcB15 hsdR4 Δ(lac-proAB)* [F' *traD36 proAB* $lacI^qZ$ ΔM15]	Yanisch-Perron *et al.*, 1985
DT1156	HB101	*pro leu thi lacY hsdR end recA rpsL20 ara-14 galK2 xyl-5 mtl-1 supE44* Str^r	Bolivar and Backman, 1979
DT1325	JE2571-4	Pil^- Fla^-, spectinomycin resistant	Bradley et al., 1980
DT2544	BB4	*supF58 supE44 hsdR514 galK2 galT22 trpR55 metB1 tonA* Δ*lac*U169 F' [*proAB*$^+$ *lacI*^{q} *lacZ*ΔM15 Tn*10*(*tet*^{r})]	Sambrook *et al.*, 1989
DT2171	DH5α	*supE44* Δ*lac*U169 (*ϕ80 lacZ* ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Hanahan, 1983
DT2517	*E. coli*B/R	(ATCC11143) radiation resistant *E. coli* B	Demerec, 1951
DT176	RG176	*E. coli* C strain "C-1a" nalidixic acid resistant	Taylor and Grant, 1977
DT2545	*E. coli* W	(ATCC9637) wild type	Davis and Mingioli, 1950
DT1098	JM83	*ara* Δ(*lac-proAB*) *rpsL*(Str^r) (*ϕ80 lacZ*ΔM15)	Yanisch-Perron *et al.*, 1985

Table 5. Bacteria Other Than *Escherichia coli* Used for PCR Amplification in This Study

Strain No.	Strain	Source or Reference
DT37	*Salmonella typhimurium* LT2	Dave Botstein[a]
DT1955	*Shigella sonnei*	Maher & Taylor, 1993
DT1957	*Klebsiella pneumoniae*	Maher & Taylor, 1993
DT1958	*Enterobacter cloacae*	Lab collection
DT1959	*Arizona*	Lab collection
DT1960	*Citrobacter*	Lab collection
DT1963	*Proteus vulgaris*	Lab collection
N/A	*Haemophilus influenzae*	Lab collection
DT82	*Pseudomonas aeruginosa*	L.E. Bryan[b]
UA580	*Campylobacter jejuni*	Lab collection
N/A	*Corynebacterium diphtheriae*	Lab collection
N/A	*Staphylococcus aureus*	Lab collection

[a] Dave Botstein, MIT (1977).

[b] L.E. Bryan, University of Calgary.

Table 6. *Escherichia coli* Deletion Mutants Used in This study

Strain No.	Strain	Chromosomal Marker	Deletion Region[a]	Origin	Reference
DT2512	PK1427	*trpR trpA*9605am *his*-29am *ilv pro*-2 *arg*-427 *thyA deoB* or *deoC tsx* IN(*rrnD-rrnE*)1 *lac* (probably *lacY*)	Δ(kb175-kb247)	Peter Kuempel	Henson *et al.*, 1984
DT2513	PK1439	*trpR trpA*9605am *his*-29am *ilv pro*-2 *arg*-427 *thyA deoB* or *deoC tsx*	Δ(kb150-kb290)	Peter Kuempel	personal communication

[a] Refer to the Bouche restriction map (Bouche, 1982).

2.3 Plasmid DNA Isolation

Plasmid DNA was isolated from many different cultures of plasmid-containing bacteria by the alkaline lysis method modified from the Birnboim and Doly (1979) method of plasmid DNA preparation. Plasmid-containing bacteria were lysed by treatment with sodium dodecyl sulfate (SDS) and NaOH. Bacterial proteins and DNA were then denatured by SDS and NaOH, respectively. Neutralization was followed by addition of sodium acetate, which caused the plasmid DNA to reanneal rapidly. Most of the chromosomal DNA and bacterial proteins remained denatured and were precipitated by forming a complex with salt. They were removed by centrifugation. The reannealed plasmid DNA was then concentrated by ethanol precipation. In large preparations, the plasmid DNA was further purified by cesium chloride (CsCl)/ethidium bromide density gradients (Whiteley and Taylor, 1983).

2.3.1 Minipreps of plasmid DNA

A volume of 5 ml BHI broth containing an appropriate antibiotic was innoculated with a single colony of plasmid-containing bacteria and was grown at 37^{o}C with shaking overnight. A 1.5 ml volume of the culture was pelleted in an eppendorf tube and was resuspended in 100 µl of solution I (0.25M Tris, 50mM Glucose, 10mM EDTA, pH 8.0) with lysozyme 2 to 4 mg/ml and then was left at room temperature for 10 min. A volume of 200 µl of solution II (0.2N NaOH, 1% SDS) was added and the cell suspension mixed well by inverting the tube a few times After cells were completely lysed, 150 µl of solution III (3M NaOac, pH4.8) was added and the suspension throughly mixed by vortexing. The mixture was incubated on ice for 20 min, subjected to centrifugation at 14,000 rpm for 10 min, and the supernatant transferred into a new eppendorf tube. Plasmid DNA was precipitated by adding 2.5 volumes of ice-cold ethanol and was pelleted by centrifugation. The dried pellet was dissolved in 30 to 50 µl of TE buffer (10mM Tris·HCl, pH 8.0; 1mM EDTA, pH 8.0).

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DT2513	PK1439	*trpR trpA*9605am *his*-29am *ilv pro*-2 *arg*-427 *thyA deoB* or *deoC tsx*	Δ(kb150-kb290)	Peter Kuempel	personal communication

[a] Refer to the Bouche restriction map (Bouche, 1982).

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2.3.2 Large-scale Preparation of Plasmid DNA

The method for large-scale preparation of plasmid DNA was originally described by Birnboim and Doly (1979) and was applied here as a modification (Whelan, 1992). This method was used to generate a large amount of high-quality plasmid DNA. Crude lysates enriched in plasmid DNA were made from bacterial cells grown in liquid culture. Then the plasmid DNA was purified by removal of both RNA and chromosomal DNA. This procedure is as fellows.

Cells were grown overnight in 250 ml of BHI broth at 37°C and were pelleted in a centrifuge plastic bottle (Sorvall) in a Beckman J2-21 centrifuge with a JA-14 rotor at 5,000 rpm for 7 min at 4°C. The supernatant was discarded. The pellet was loosened using the end of a micropipette from the side of the bottle and was resuspended in 20 ml of Solution I with lysozyme 2 to 4 mg/ml by gentle swirling for large plasmid and by vigorously shaking or vortexing for recombinant plasmid clones. The bottle was incubated on ice for 30 min. A volume of 40 ml of solution II was added and mixed well to achieve as clearly as possible. A 30 ml volume of solution III was added and throughly mixed by inverting the bottle several times (very gently for large plasmids) to yield a white precipitate. Incubation of the bottle was then continued on ice for 45 min. The bottle was centrifuged at 9,000 rpm for 30 min at 4°C. The supernatant was transferred into a new bottle. Any floating white precipitates were removed using a Pasteur pipette. An equal volume of isopropanol was then added. The bottle was inverted several times and was placed at -20°C for two hours or more to precipitate DNA, then spun at 10,000 rpm and 4°C for 10 min. The pellet was drained completely and was resuspended in 10 ml of TE with RNAase at 0.25 µg/ml. 10 g of CsCl was dissolved in the resuspended liquid containing the crude plasmid DNA. The preparation was then transferred into a Beckman Quick-seal tube with 200 µl of 10 mg/ml ethidium

bromide by using a needle. The tube was sealed and centrifuged at 55,000 rpm in 70.1Ti rotor for 18 to 24 hours at 20°C. After removing the tube from the rotor, a hole was made using a needle at the top of the tube. The plasmid DNA band was recovered by suction using a 3 ml syringe with another needle attached and collected into a clear Nalgene tube containing at least an equal volume of CsCl-saturated isopropanol. The tube was inverted several times. The top layer which was usually pink, was removed and another equal volume of CsCl-saturated isopropanol was added to the tube. This step was repeated until the pink color disappeared. The bottom layer was then moved into an opaque Nalgene tube and was diluted with two to three volumes of solution IV (0.1 M sodium acetate, 50mM Tris-HCl, pH8.0) and precipitated with two volumes of absolute alcohol at -20°C for an hour. The DNA pelleted was dissolved in an appropriate volume of sterile TE or ddH_2O depending upon the amount of the DNA isolated and was stored in an Eppendorf tube at 4°C.

2.4 Restriction Endonuclease Digestion

Restriction enzymes used in this study are listed in Appendix II along with the conditions used. They were obtained from Boehringer Mannheim Biochemicals, Canada, Ltd. (BMC), Montreal, Quebec, or Bethesda Research Laboratories (BRL), Canada, Ltd., Mississauga, Ontario. Restriction digestions were carried out according to the manufacturer's recommendations using Boehringer Mannheim SuRE/Cut buffers. Double digestions were carried out simultaneously (for two enzymes that require the same buffer) or by performing one digestion, then precipitating the DNA and then performing the second digest. Restriction digestion was terminated by the addition of 1/5 volume of bromophenol blue (BPB) loading dye (48% sucrose, 0.25% bromophenol blue, 12 mM EDTA) to DNA. Digestion products were analyzed using agarose gel electrophoresis.

2.5 Agarose Gel Electrophoresis

DNA samples were mixed with BPB loading dye and subjected to electrophoresis in a horizontal agarose gel apparatus containing a 0.5-1.2% agarose (Gibco BRL) gel in 1x Tris-Acetate-EDTA (TAE) buffer (see Appendix I) (Portnoy *et al.*, 1981). Electrophoresis was carried out at 34-110 V (constant voltage) for up to 48 hrs with periodic changes of the running buffer. Gels were stained with ethidium bromide after electrophoresis and destained with distilled water. DNA fragments were visualized under ultraviolet transillumination (260nm). Sizes of restriction digest fragments were determined with reference to bacteriophage lambda digested with *Hin*dIII or *Eco*RI.

2.6 Extraction of DNA Fragments from Low Melting Point Agarose

Restriction endonuclease digests were subjected to electrophoresis as described above except that low-melting-point agarose (Gibco BRL) was used. After staining with ethidium bromide, the desired DNA band(s) was excised over the ultraviolet (UV) transIluminator (300nm). The agarose block was placed in a 1.5 ml Eppendorf tube and immersed in a final volume of 0.5 ml TE buffer. The DNA was extracted from the low-melting-point agrarose by heating at 65^{o}C for 10 minutes. An equal volume of Tris-equilibrated phenol was added, the mixture was vortexed and then centrifuged for 2 minutes in a microfuge at 14,000 rpm. The aqueous phase (top) was carefully collected in a new Eppendorf tube, mixed with an equal volume of choloroform and subjected to centrifugation. The aqueous phase (top) was collected in a new tube and precipitated with 2.5 volumes of 95% ethanol at -20^{o}C. Afer the pellet was completely dry, it was dissolved in an appropriate volume of TE buffer (depending on the amount of DNA).

2.7 Minimum Inhibitory Concentration (MIC) Determination

Minimum Inhibitory Concentration (MIC) is the minimal concentration of an antimicrobial agent required to inhibit or kill a microorganism. Serial dilutions of the antimicrobial agent in an agar (agar dilution method) or broth (broth dilution method) medium are inoculated with the organism and incubated. The MIC is determined as the lowest concentration without visible growth. In our study, the agar dilution method (Washington, 1985) was used because of its convenience for testing a number of strains simultaneously. Bacterial strains to be tested were grown overnight in Brain heart infusion (BHI) (OXOID) broth at 37°C with shaking, then diluted 1 in 100 in BHI broth. Volumes of 10 µl were spotted onto a series of Mueller Hinton (MH)(OXOID), BHI agar (OXOID) or Miller's LB agar (BBL) plates containing twofold dilutions of an antimicrobial agent (e.g. potassium tellurite, K_2TeO_3). Plates were then incubated overnight at 37°C. The lowest concentration of the antimicrobial agent which inhibited growth was determined to be the MIC.

2.8 Procedure for Radioactive Labeling of DNA Using Nick Translation

DNA fragments from cleaned PCR products or from low-melting-point agarose by phenol/chloroform extraction were labeled with [α-^{32}P] dCTP by using a commercial Nick Translation System (Gibco. BRL) kit. Labeling was performed according to the recommended procedure by the manufacturer except that labeled DNA was washed with 70% ethanol after precipitation and that if the amount of DNA was very small, 5-10 µg of yeast tRNA was sometimes added to seed the precipitation. The pellet of the labeled DNA was then dissolved in 400 µl of TE buffer. The degree of labelling was detected in the Beckman LS 6800 scintillation counter. One million CPM of the labelled DNA was used for one hybridization.

2.9 Preparation of Dot Blots

DNA was isolated as previously described. DNA concentrations were determined by measurement of the absorbance at 260 nm in a spectrophotometer (1 OD = 50 μg/ml DNA) or determined by comparison with the intensity of a DNA band of known concentration in an agarose gel.

A sheet of nitrocellulose membrane (Trans-Blot^R Transfer Medium, Bio-Rad) was cut to an appropriate size. It was completely soaked with ddH_2O and was then soaked in 10 x SSC (1.5M NaCl, 0.5M sodium citrate, pH7.0) for 30 min with gentle shaking. The filter was drained and applied to a dot blot apparatus. One μg of plasmid DNA or ten μg of genomic DNA was diluted in 20 μl ddH_2O, which was then loaded onto the nitrocellulose filter under vacuum. DNA was denatured and neutralized by placing the filter for 10 min each onto a sheet of Whatmann 3MM Chromatography Paper soaked with denaturing solution (1.5M NaCl, 0.5M NaOH) and neutralizing solution (2M NaCl, 1M Tris-HCl, pH 5.5), respectively. The filter was finally placed onto a sheet of Whatmann 3MM Chromatography Paper wet with 2xSSC. Then it was air dried at room temperature and baked at 65^{o}C overnight.

2.10 Southern Transfer

After DNA was digested with endonucleases and subjected to gel electrophoresis as previously described, DNA was denatured by immersing the gel in denaturing solution (1.5M NaCl, 0.5M NaOH) for 60 min with gentle shaking. Denaturing solution was poured off and replaced with neutralizing solution (2M NaCl, 1M Tris-HCl, pH 5.5). Neutralization was continued for 90 min in the same way with a change of the solution every 30 min. Large DNA fragments were nicked with UV to facilitate transfer prior to

the steps of denaturation and neutralization. DNA then was transferred to Hybridization Transfer Membrane (Micron Separations Inc., MSI) by the method of Southern (1975). After transfer, the membrane was soaked in 2 x SSC (0.3M NaCl, 0.1M sodium citrate, pH7.0) for 10 min to remove excess salt. It was air dried and baked overnight at 65^{O}C.

2.11 DNA Hybridization

Hybridization of ^{32}P-labelled DNA fragment to the DNA on nitrocellulose filters was performed as described by Portnoy *et al* (1981). Nitrocellulose filters were preincubated for at least one hour at 42^{O}C with 5 ~ 10 ml of pre-incubation mixture [50% formamide; 5 x SSC; 0.1% sodium dodecyl sulfate (SDS); 1 mM EDTA; 0.02% ficoll; 0.02% bovine serum albumin (BSA); 0.02% polyvinyl pyrrolidone (PVP)] and 750 µg denatured herring sperm DNA in a heat-sealable plastic bag.

The radioactively-labelled DNA probe was denatured by boiling for 5 min, chilled in iced-water and added to the plastic bag. Incubation was continued at 42^{O}C overnight. After hybridization, the filters were soaked in 0.1 x SSC; 0.1% SDS and 1 mM EDTA) for 30 min at room temperature and then washed twice with 2 x SSC; 0.1% SDS and 1 mM EDTA at 65^{O}C for 45 min. They were then rinsed quickly with 2 x SSC, drained and wrapped in plastic wrap. They were exposed to X-ray film (Kodak X-OMAT AR) for various periods of time at -80^{O}C according to the amount of radioactivity present.

2.12 Isolation of Chromosomal DNA

Fresh cultures of bacteria strains were prepared on agar plates. A full loop of cells were suspended in 150 µl of TES (50 mM Tris, pH 8.0; 10 mM EDTA; 25% sucrose) and 125 µg of lysozyme was added. The suspension was incubated at 37^{O}C for 30 min, and then mixed with 100 µl of phenol saturated with 0.1 M Tris pH 8.0. Effective

mixing was obtained by vortexing for 2-3 min. A volume of 100 µl chloroform was added and the suspension mixed well by vortexing. Centrifugation was carried out at 14,000 rpm for 5 min at room temperature. The upper phase was carefully removed to an Eppendorf tube. DNA was precipitated by adding two volumes of 95% cold ethanol and the pellet redissolved in 200 µl TE buffer.

2.13 Titration of Lambda Phage Stock

A 5 ml volume of an overnight culture of *E. coli* BB4, a lambda-sensitive strain, was grown to saturation at 37°C in LB medium containing 10 mM $MgSO_4$ and 0.2% maltose. Dilutions of 100-fold of the phage lysate were made in SM (see Section 2.1) and 0.1 ml of each dilution was added to one tube containing 0.3 ml of the *E. coli* saturated culture. The *E. coli*/phage mixtures were incubated at room temperature for 20 min so that the phage could absorb to the *E. coli* cells. The mixtures were incubated at 37°C for 10 min and so that the phages could inject their DNA into the cells. LB-top agar (see section 2.1) was melt and cooled to 45 to 50°C. A volume of 2.5 ml of melted top agar was added to each tube containing *E. coli* /phage mixtures. The content of each tube was mixed by vortexing lightly and poured onto a 10 mM $MgSO_4$-LB plate (see Section 2.1). The agar was spread over the entire surface of the plate by tilting the plate gently. The plates were incubated at 37°C for 12 hours. One of the dilution plates, which was not too crowded with plaques, was chosen. The number of plaques was counted and was used to determine the number of viable phage in the starting stock suspension.

2.14 Preparation of Lambda Phage Lysate

A saturated culture of *E. coli* BB4 was made as described above. Phage particles were absorbed to the bacteria by combining 0.1 ml of 10^7 PFU/ml eluted phage with 0.1 ml of saturated culture and 0.1 ml of 10 mM $MgCl_2$/ 10 mM $CaCl_2$ solution and incubating in a 37°C water bath for 20 min. The mixture was inoculated into 5 ml of 2 x YT/MT medium in a Nalgene tube and was then incubated at 37°C with rotation until lysis occurs (usually 6-8 hours). The culture was harvested immediately lysis was observed. Any remaining viable cells were lysed by adding 0.5 ml of chloroform. Chloroform was removed from the bottom of the tube using a Pasteur pipette. The lysate was centrifuged at 6,000 rpm and 4°C for 10 min in a Beckman J2-21 centrifuge with a JA-20 rotor to pellet the cell debris.

2.15 Isolation and Purification of Lambda Phage DNA

A clarified phage supernatant was prepared as described in the above section. Lambda phage DNA was extracted and purified by using a Sephaglas™ PhagePrepKit as recommended by the manufacturers.

2.16 Polymerase Chain Reaction (PCR)

The oligonucleotide primers with their sequences are shown in Table 7. The primers RTUPCR7 and RTUPCR8 were used to amplify the whole open reading frame of the *tehA* gene, which is devoid of the ribosome binding site (Shine-Dalgarno sequence, "SD") and is 1018bp in length. The primers RTUPCR9 and RTUPCR10 were used to amplify the complete portion of the gene *tehB*, which is 621bp in length. RTUPCR7 and RTUPCR10 were used to amplify *tehAB*, which is 1610bp in length. The primers 23S-1 and 23S-2 gave an amplified PCR product of 603bp, which

constitutes a portion of the 23S-rRNA gene (Taylor *et al.*, 1992). Amplification of the 23S-rRNA gene was used as a positive control for PCR reactions using genomic DNA as a template. The amount of each primer used in one PCR reaction was 100 pmoles.

Taq DNA Pol I was obtained either from Dept. of Biochemistry, U. of A. or from Promega Corporation. The enzyme obtained from the latter source was used as recommended by the supplier, whereas the enzyme from the former source was used in a buffer containing 1.25 mM dNTP; 50mM HEPES pH7.9; 1.5 mM $MgCl_2$; 50mM KCl.

The chromosomal DNA templates used in this study are shown in Tables 3 and 5. They were isolated from a variety of bacteria using the method described in section 2.12. The DNA from plasmid pDT1364 and pUC8 were prepared by the large-scale isolation procedure and CsCl/ethidium bromide ultracentrifugation gradient as described in Section 2.3.2. The plasmid DNA obtained this way still had some degree of chromosomal contamination. For further purification, plasmid DNA was linearized to give a single band on gel electrophoresis. The plasmid DNA of pDT1364 and pUC8 was digested with either of the two restriction endonucleases, *Bam*HI and *Eco*RI. These enzymes were chosen because they cut both plasmids once. Then the digests were subjected to agarose gel electrophoresis. The single DNA band of each plasmid digest was excised from a gel and was further purified by the method described in Section 2.6. In this way, the possible contamination of plasmid DNA isolates with chromosomal DNA was eliminated. The DNA from each sample was subjected to triplicate PCR reactions amplifying *tehA*, *tehB* and 23S rRNA gene. If both *tehA* and *tehB* were amplified from one DNA sample, the DNA was also subjected to the fourth PCR to amplify *tehAB*. A negative control reaction containing the same PCR mixture, except using ddH_2O instead

of a DNA template, was also performed in parallel. This was done to determine if there was any contamination during setting up PCR.

For each PCR, 0.5 μg of plasmid DNA or 2.5 μg of chromosomal DNA was used. PCR reactions were carried out in 100 μl volume using Perkin-Elmer DNA Thermal Cycler 480, with the following cycling profiles: for *tehA* and *tehB*, 94°C for 1 min, 55°C for 2 min and 72°C for 2 min for 30 cycles, then 72°C for 10 min; for *tehAB* and 23SrRNA genes, 94°C for 1 min, 55°C for 1 min 40 sec and 72°C for 3 min for 20 cycles, 72°C for 20 min. The PCR products were analyzed directly on a 1.1 % agarose gel using 10 μl of each sample.

Table 7. Primers Used in This Study

Primer[a]	Sequence[b]
RTUPCR7	5' ATATGGATCCATGATCAGCGATAAAGTGCTCAATTTG 3'
RTUPCR8	5' ATATGTCGACTGAATTCATTCTTTGTCCTCTGCTTTC 3'
RTUPCR9	5' ATATGAATTCATGATCATTCGTGACGAAAAC 3'
RTUPCR10	5' ATATGTCGACTGATATCTCATTTTTTACGTGCCAGCATC 3'
23S-1	5' GTCGGGTAAGTTCCGACCT 3'
23S-2	5' GGCGAACAGCCATACCCTT 3'

[a] RTUPCR7 and RTUPCR8 were used to amplify the whole open reading frame of the *teh*A gene. RTUPCR9 and RTUPCR10 were used to amplify the complete portion of the gene *teh*B. RTUPCR7 and RTUPCR10 were used to amplify *teh*AB. The primers 23S-1 and 23S-2 amplified a portion of the 23S-rRNA gene.

[b] The underlined nucleotides represent sequences homologous to pDT1364.

3. RESULTS

Part A. Incidence of Tellurite Resistance Determinants among Plasmids of Different Incompatibility Groups

3.1. MICs of Tellurite, Arsenite and Arsenate Mediated by a Variety of Te^r Plasmids from different Inc groups

A variety of wild-type plasmids from different Inc groups (Table 2), as well as the recombinant plasmids pDT1364, pDT1555 and pMJ606 (see Table 3), were tested to determine MICs of potassium tellurite (K_2TeO_3), sodium arsenite ($NaAsO_2$) and sodium arsenate ($Na_2HAsO_4 \cdot 7H_2O$). Most of these plasmids have been found to encode resistance to potassium tellurite. Strains of *E. coli* containing these plasmids were tested to determine their levels of resistance to tellurite. MICs of arsenic oxy-anions including arsenite and arsenate were also determined in order to define the specificity of the Te^r determinants.

MICs were determined as previously described (Section 2.7). The results are shown in Table 8. All the IncHII plasmid-containing isolates of *E. coli* K-12 hosts demonstrated a high level of Te^r with MICs of tellurite at least 512 μg/ml. All the IncHI2 plasmid-bearing strains tested also demonstrated a high level of Te^r (MICs were 512μg/ml or higher). An exception was the plasmid R476b which had a MIC of tellurite 4 μg/ml and was used as a tellurite-sensitive (Te^s) control plasmid. The MIC mediated by IncHI1 plasmid pRG1251 was 2 μg/ml which is the same as the MIC for both the plasmid-free strain of *E. coli* JM83 and the vector-control plasmid pBR322. All the IncPα plasmids also had very low MICs (2 μg/ml), whereas all the P2 plasmids were

shown to mediate MICs of tellurite at 256 to 512 μg/ml. Plasmids RAS5002, RAS5003, RAS5005, RAS5009 and RAS5012 were found to be highly resistant to tellurite with MICs at 1024 μg/ml. However their Inc groups have not yet been determined. The MIC mediated by the recombinant plasmids pDT1555 and pMJ606 was 1024 μg/ml, whereas with pDT1364, the MIC was lower at about 128 μg/ml.

The plasmid-free strain *E. coli* JM83 and the vector-control plasmid pBR322 as well as plasmid pRG1251 (HI1) and all the IncPα plasmids tested were shown to be sensitive to both tellurite and the two arsenicals. In contrast the Te^S control plasmid R476b specified a very high MIC of arsenite and arsenate. Many of the Te^r plasmids including pMG56 (P2), pMG64 (P2), CAM (P2), pHH1457 (HII), RAS5007 (HI2), TP116 (HI2), R1022 (HI2) and the five plasmids of undetermined incompatibility did not encode resistance to either of the arsenic oxy-anions. *E. coli* harboring any of the three Te^r plasmid clones were also shown to be sensitive to the arsenic oxy-anions. However, many Te^r plasmids were found to encode resistance to both arsenite and arsenate. These include pMG53 (P2), pHH1532b-1 (HII), pHH1457-b (HII), R826 (HI2), R826-1 (HI2), R828 (HI2), pWR23 (HI2), R478(HI2), pJT1(HI2), pMG223(HI2), pMG224(HI2), pMG225 (HI2) and MIP233 (HI3). They were shown to specify high MICs of arsenite at 1024 to 2048 μg/ml and arsenate at 32 to 128 mg/ml. Plasmid pAS-251-2 (HI2), which specified a MIC of tellurite of 1024 μg/ml, was found to encode a MIC of arsenite at 1024 μg/ml but a MIC of arsenate at 8 mg/ml.

Table 8. MICs of Tellurite, Arsenite and Arsenate Encoded by Plasmids and Cloned Derivatives

DT No.	Host[a]	Plasmid	Inc[b]	MIC[c] Tellurite	Arsenite	Arsenate
1364	JM83	pDT1364	N/A	128	512	8
1555	JM83	pDT1555	N/A	1024	512	8
1798	AB1157	pMJ606	N/A	1024	512	16
481	RR1	pBR322	N/A	2	512	16
1098	JM83	None	N/A	2	512	8
286	RG192	R26	Pα	2	512	4
287	RG192	RP1	Pα	2	512	4
292	RG192	RP638	Pα	2	512	4
293	RG192	RP527	Pα	2	512	4
294	RG192	R1033	Pα	2	512	4
295	RG192	Rm16b	Pα	2	512	4
298	RG192	R702	Pα	2	512	4
561	RG192	R1033	Pα	2	512	4
82	*P. aeruginosa* 280	None	N/A	4	256	4
83	*P. aeruginosa* 280	R931	P2	256	256	4
84	*P. aeruginosa* 280	R130	P2	512	256	4
2203	*P. aeruginosa* PU21	pMG6	P2	512	512	8
2204	*P. aeruginosa* PAO38	pMG31	P2	512	512	8
2199	*P. aeruginosa* PAO38	pMG53	P2	512	2048	128
2200	*P. aeruginosa* PAO38	pMG56	P2	256	512	8
2205	*P. aeruginosa* PAO38	pMG64	P2	512	512	8
2207	*P. aeruginosa* PU21	CAM	P2	>1024	512	8
975	RG2137	pHH1508a	HII	>1024	512	16
993	J53-1	pHH1532b-1	HII	>1024	1024	64

Table 8. continued.

DT No.	Host[a]	Plasmid	Inc[b]	MIC[c] Tellurite	Arsenite	Arsenate
994	J53-1	pHH1457	HII	1024	512	16
997	J62-1	pHH1457-b	HII	512	1024	32
1	JM53	R476b	HI2	4	2048	128
2	J53-2	R826	HI2	>1024	2048	128
3	J53-2	R826-1	HI2	>1024	2048	128
4	J53-2	R828	HI2	1024	2048	64
64	*E. coli* C	pWR23	HI2	>1024	4096	128
65	*E. coli* C	MIP235	HI2	512	2048	128
71	AB1932-1	RAS 5007	HI2	1024	512	16
78	RG192	TP116	HI2	>1024	512	8
109	RG192	R478	HI2	>1024	2048	128
135	RG192	pAS-251-2	HI2	1024	1024	8
416	RG192	pJT1	HI2	>1024	2048	32
447	J53-1	R1022	HI2	512	512	8
2206	J53-2	pMG223	HI2	>1024	1024	64
2197	J53-2	pMG224	HI2	>1024	1024	64
2198	J53-2	pMG225	HI2	>1024	1024	64
63	*E. coli* C	MIP233	HI3	>1024	512	32
106	RG192	pRG1251	HI1	2	512	8
68	AB1932-1	RAS5002	ND	1024	512	16
69	AB1932-1	RAS5003	ND	1024	512	16
70	AB1932-1	RAS5005	ND	1024	512	16
72	AB1932-1	RAS5009	ND	1024	512	16
73	AB1932-1	RAS5012	ND	1024	512	16

[a] Where host not shown, the strain is *E. coli* K-12.

[b] Plasmid Inc group refers to the plasmid itself; N/A, not applicable; ND, not determined

[c] The unit of MIC used for both tellurite and arsenite is μg/ml; whereas the unit for arsenate is mg/ml.

3.2. MICs of Arsenite, Arsenate and Tellurite, Encoded by the Arsenical Resistance (*ars*) Operon of the Resistance Plasmid R773

MICs of tellurite, arsenite and arsenate were determined as described before. The MIC of arsenate mediated by the plasmid pUM3 (*arsABC*) (Mobley *et al.*, 1983) was 64 mg/ml, whereas with pAB100 (*arsRAB*) (B. P. Rosen 1991, Personal communication), pUM11ΔBC (*arsA*) (B. P. Rosen 1991, Personal communication) and pWSU2 (*arsC*) (Rosen *et al.*, 1988), the MIC was 4, 8 and 16 mg/ml, respectively. The MIC of arsenite mediated by the plasmids pUM3 (*arsABC*) and pAB100 (*arsRAB*) was 4096 μg/ml, whereas with pUM11ΔBC (*arsA*) and pWSU2 (*arsC*), the MIC was much lower at 512 μg/ml. The MIC of potassium tellurite encoded by these plasmids containing *ars* genes was also determined. The MIC of tellurite mediated by the recombinant plasmids pAB100 (*arsRAB*), pUM11ΔBC (*arsA*) and pWSU2 (*arsC*) was about 4 μg/ml, whereas with the plasmid pUM3 (*arsABC*), the MIC was 64-128 μg/ml (Turner *et al.*, 1992).

3.3. DNA-DNA Dot Blot Hybridization of a Variety of Plasmids from Different Inc Groups Using Different Te^r determinants as Probes

DNAs from a variety of plasmids of different incompatibility groups (Tables 2 and 3) were isolated using methods described in Section 2.3 and were applied to a sheet of nitrocellulose filter membrane using a dot-blot apparatus. After denaturing and neutralizing the DNA, the blots were subjected to hybridization with DNA probes. The Te^r determinants (IncPα, HI2 and pDT1364) were isolated from purified plasmids pDT1555, pMJ606 and pDT1364, respectively. The *ars* determinant was isolated from the plasmid pUM3. Each of these determinants was then ^{32}-P-radiolabelled by nick-translation and was used to hybridize to the blots.

The Te^r determinant from an IncPα plasmid hybridized only with the IncPα plasmids tested. None of the other probes hybridized to IncPα plasmids. The *ars* determinant (pUM3) did not show any cross-hybridization with any of the Te^r determinants or plasmids of IncHI2, HII or P groups. The IncHI2 Te^r determinant (pMER610) hybridized with all IncHI2 plasmids except R476b. It also hybridized with all the IncHII plasmids as well as all the unclassified Te^r plasmids (Table 2).

The Te^r determinant (6.8kb) of pDT1364, which was believed to originate from the large IncHII plasmid pHH1508a, was shown to hybridize to a variety of plasmids within different Inc groups as well as the plasmid pHH1508a haboured in the strain of *E. coli* J53-1 (assigned as DT898). However, it also hybridized with *E. coli* J53-1 chromosomal DNA and did not hybridize with DT975 = RG2137(pHH1508a) consistently. Thus two possible explanations were proposed. Firstly, if the pDT1364 insert was from pHH1508a, then the plasmid pHH1508a initially might have had two Te^r determinants and the plasmid in DT975 might have undergone a rearrangment to lose one of the two Te^r determinants. Secondly, the insert present in pDT1364 might have

originated from the *E. coli* chromosome due to chromosomal contamination in the preparation of plasmid DNA. To test these hypotheses, the restriction patterns of pHH1508a from strains of DT898 and DT975 were compared to each other and to that obtained previously (Yan, Ph.D thesis, 1986).

3.4. Restriction Endonuclease Digestion of pHH1508a DNA with *Xba*I and *Xho*I

To test the first hypothesis proposed to explain the results of dot-blot hybridization by the ^{32}-P-radiolabelled pDT1364 insert, the restriction digestion of pHH1508a from strains DT898 and DT975 as well as one original pHH1508a isolate P78 = J53-1 (pHH1508a) was carried out. The restriction enzymes *Xba*I and *Xho*I which cut pHH1508a into a reasonable number of fragments and had been used in the construction of the pHH1508a restriction map (Yan and Taylor, 1987; Yan, M.Sc. thesis, 1986) were used to digest pHH1508a. The plasmid DNA was isolated from the three different strains by the large-scale preparation and was purified by cesium chloride (CsCl)/ethidium bromide density gradients (Section 2.3.2.) and was subjected to digestions. The digests were then subjected to electrophoresis for 17 hours using 0.5% agaros gels in 1x TAE buffer (Appendix I) under constant voltage of 46V. The restriction patterns of *Xba*I and *Xho*I are shown in FIG. 3. Lanes A and K are λ phage DNA cut with *Hin*dIII and *Eco*RI used as size markers. Lanes B, C and D are the samples of *Xba*I digestion; lanes H, I and J are the samples of *Xho*I digestion; lanes E, F, and G are *Xba*I / *Xho*I double digestion. Comparison of the digestion patterns of the three pHH1508a isolates to the one that was obtained previously (Yan, Ph.D thesis, 1986) shows obvious changes in the restriction patterns of DT975 with *Xba*I, *Xho*I and *Xba*I / *Xho*I double digestion, whereas no changes were observed in either DT898 or P78.

FIG. 3 Agarose gel electrophoresis of pHH1508a isolated from different cultures and digested with *Xba*I, *Xba*I / *Xho*I and *Xho*I. Digests were run on a 0.5% agaros gel in 1x TAE buffer for 17 hours under constant voltage at 46V. Lanes A through K show ethidium bromide-stained gel with *Xba*I fragments in lanes B, C and D; with *Xba*I / *Xho*I fragments in lanes E, F, and G and with *Xho*I fragments in lanes H, I and J. Lanes A and K are λ phage DNA cut with *Hin*dIII and *Eco*RI, respectively. The molecular sizes of the *Hin*dIII fragments are shown on both sides in kilobases (kb).

(A) λ phage DNA digested with *Hin*dIII

(B) pHH1508a from DT898 digested with *Xba*I

(C) pHH1508a from DT975 digested with *Xba*I

(D) pHH1508a from P78 digested with *Xba*I

(E) pHH1508a from DT898 digested with *Xba*I / *Xho*I

(F) pHH1508a from DT975 digested with *Xba*I / *Xho*I

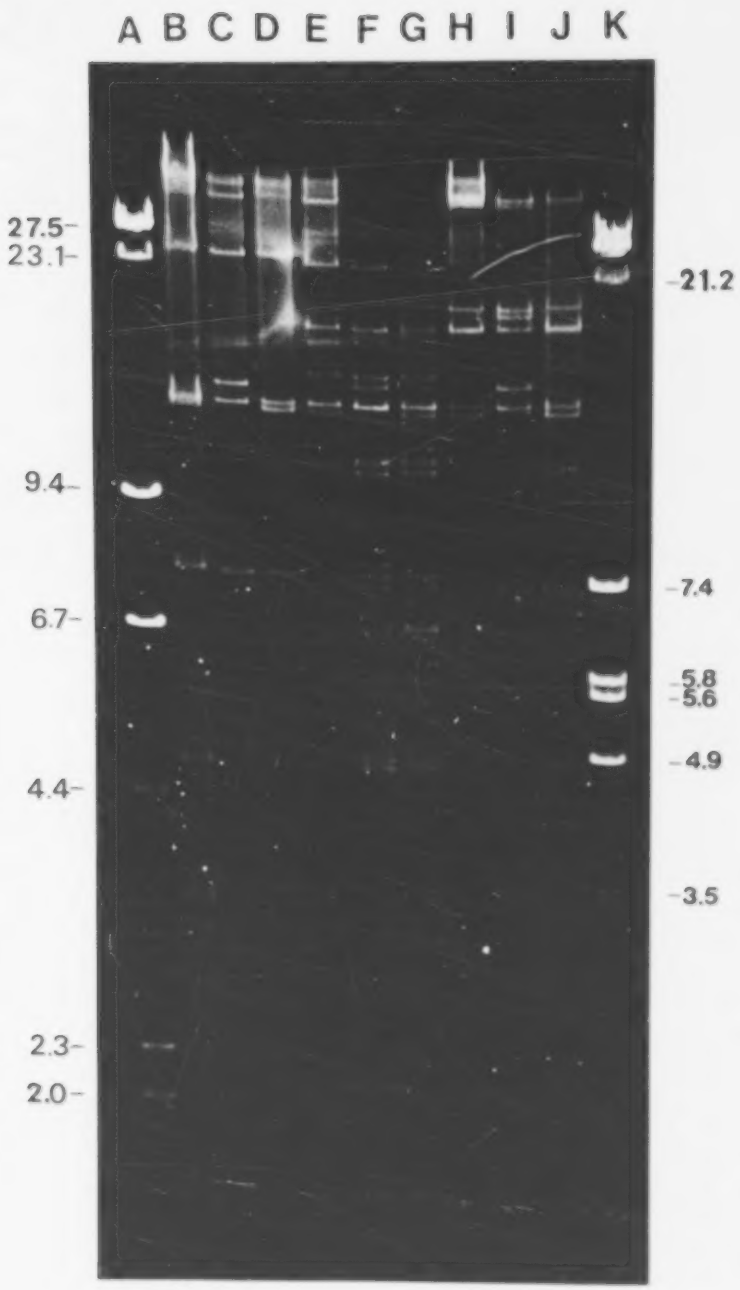
(G) pHH1508a from P78 digested with *Xba*I / *Xho*I

(H) pHH1508a from DT898 digested with *Xho*I

(I) pHH1508a from DT975 digested with *Xho*I

(J) pHH1508a from P78 digested with *Xho*I

(K) λ phage DNA digested with *Eco*RI


A B C D E F G H I J K
27.5
23.1
9.4
6.7
4.4
2.3
2.0
21.2
7.4
5.8
5.6
4.9
3.5

3.5. Southern Blot Hybridization of Te^r Plasmids from Different Inc Groups Using Different Te^r determinants as Probes

In order to confirm the results obtained by dot-blot hybridization, and also to determine if plasmids might have more than one Te^r determinant, Southern hybridizations were carried out. One of the wild-type plasmids from each Inc group, i.e. Pα, HI2 or HII was chosen as a representative. They were digested with an appropriate endonuclease, for RP1 with *Sma*I; for R478 and pHH1457b with *Xba*I. Digestions of pDT1364 with *Sal*I, pMJ606 with *Pst*I / *Bgl*II and pDT1555 with *Bam*HI / *Hin*dIII were used as positive controls for hybridizations. The digests were subjected to electrophoresis and were transferred as described in Section 2.10 to nitrocellulose membrane. The blot was then probed with the different ^{32}-P-radiolabelled Te^r determinants (IncPα: 3.1-kb fragment from pDT1555 digested with *Bam*HI / *Hin*dIII; IncHI2: 3.6-kb fragment from pMJ606 with *Pst*I / *Bgl*II; and 6.8-kb fragment from pDT1364 with *Sal*I) respectively. The results are shown in FIG. 4. Only RP1 hybridized with the probe from the IncPα Te^r determinant, whereas R478 and pHH1457b did not (Panel a). With the probe of IncHI2 (pMJ606) Te^r determinant (Panel b), there was a strong hybridization signal in pHH1457b, a very weak signal in R478 and no hybridization signal in RP1. These results suggest that IncHI2 (pMJ606) Te^r determinant has low level homology with that in R478 and very high homology with that in pHH1457b. With the probe of , No hybridization was observed between any of the three plasmids and the probe of pDT1364 Te^r determinant (Panel c). This indicates that the pDT1364 Te^r determinant does not originate from these plasmids.

To test if pHH1508a contains two Te^r determinants and if the change in the restriction pattern of pHH1508a in DT975 accounts for the loss of one of the Te^r determinant, pHH1508a isolated from strains DT898, DT975 and P78 was digested with *Xba*I and *Xho*I. Plasmids pHH1457b and R478 were also digested with *Xba*I. The

digests were then subjected to electrophoresis, Southern transfer and hybridization with probes of pDT1364 and IncHI2 (pMJ606) Te^r determinants separately. With the probe of the pDT1364 Te^r determinant (data not shown), neither pHH1457b nor R478 hybridized. These results are consistent with the result in FIG. 4. However the pHH1508a DNA from DT898 and P78 did not hybridize either. Thus the first hypothesis that pHH1508a might have two Te^r determinants was eliminated.

With the probe of Inc HI2 (pMJ606) Te^r determinant (FIG. 5), pHH1508a from all three isolates hybridized. The hybridization signals were located at the *Xho*I "E" fragment, the *Xho*I / *Xba*I "C" fragment and the *Xba*I "B" fragment with reference to the restriction map made by Yan (1986, M.Sc. thesis). Plasmid pHH1457b also hybridized which is consistent with the result in FIG. 4. However, no hybridization signal was observed in R478, which again suggests little homology between the two IncHI2 Te^r determinants. Interestingly, when the duplicate blot was probed with the MIP233 Te^r determinant (6.3-kb fragment from MB7H5 cut with *Hin*dIII), the exact same hybridization pattern as that in FIG. 5 was observed. To confirm the hybridization data, another blot containing *Xho*I-digested pHH1508a from DT898 and DT975 as well as *Bam*HI-digested MIP233, the positive control sample, was made and probed with the MIP233 Te^r determinant. As shown in FIG. 6, a 9.8-kb fragment in *Bam*HI-digested MIP233 and a 17.5-kb fragment in both pHH1508a isolates hybridized. Thus, the two Te^r determinants, IncHI2 (pMER610) and IncHI3 (MIP233) Te^r determinants are homologous to one another.

To further confirm the limited homology between the two IncHI2 Te^r determinants present in pMJ606 and R478, the R478 Te^r determinant was isolated from a R478 subclone, pKFW6b, containing the R478 Te^r determinant (Whelan, Ph.D. thesis, 1993). The plasmid pKFW6b was digested with *EcoR*I /*Sal*I. A 6.2 kb fragment was isolated,

^{32}-P-radiolabelled by nick-translation and then used to hybridize to a blot containing digested DNA from different Te^r plasmids. As shown in FIG. 7, the probe not only hybridized with R478 but also hybridized with the 9.3 kb Te^r fragment from pHH1457b, pHH1508a, MIP233 and its Te^r subclone MB7H5. This indicates that there is homology between the R478 Te^r determinant and the Te^r determinants of pMER610 and MIP233.

FIG. 4 Southern blot hybridization of *Sma*I-digested RP1, *Xba*I-digested R478 and pHH1457b using different Te^r determinants from pMJ606 (b), pDT1364 (c) and pDT1555 (d) as probes. Lanes 1 through 6 in (a) show ethidium bromide-stained gel and lanes A through F in (b), (c) and (d) show the corresponding hybridization patterns.

Lane (1); (A) pDT1364 digested with *Sal*I

(2); (B) pMJ606 digested with *Pst*I / *Bgl*II

(3); (C) RP1 digested with *Sma*I

(4); (D) R478 digested with *Xba*I

(5); (E) pHH1457-b digested with *Xba*I

(6); (F) pDT1555 digested with *Bam*HI / *Hin*dIII

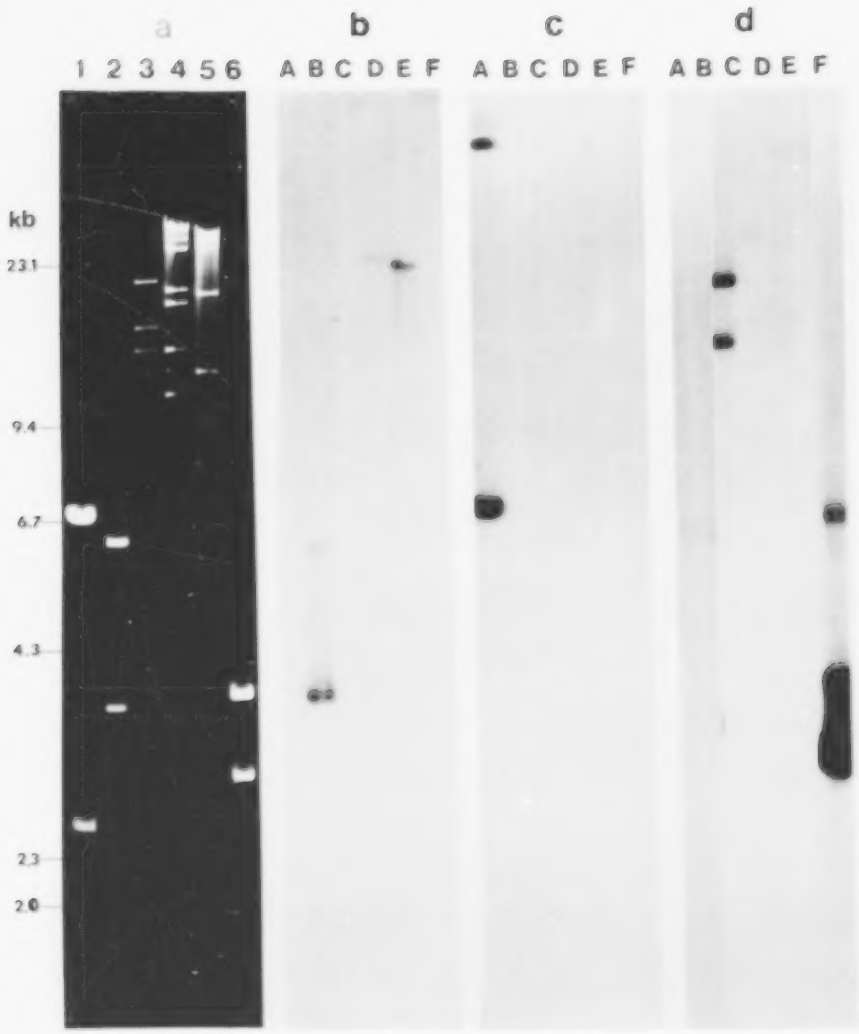

a
b
c
d
1 2 3 4 5 6
A B C D E F
A B C D E F
A B C D E F
kb
23.1
9.4
6.7
4.3
2.3
2.0

FIG. 5 Southern blot hybridization of pHH1508a isolated from different strains as well as R478 and pHH1457b using the Te^r determinant from pMJ606 as a probe. Lanes 1 through 11 in (a) show ethidium bromide-stained gel and lanes A through K in (b) show the corresponding hybridization patterns. Lane (λ) is λ phage DNA digested with *Hin*dIII.

Lane (1); (A) pDT1364 digested with *Sal*I

(2); (B) pMJ606 digested with *Pst*I / *Bgl*II

(3); (C) pHH1508a from DT975 digested with *Xba*I

(4); (D) pHH1508a from DT975 digested with *Xba*I / *Xho*I

(5); (E) pHH1508a from DT975 digested with *Xho*I

(6); (F) pHH1508a from DT898 digested with *Xba*I

(7); (G) pHH1508a from DT898 digested with *Xba*I / *Xho*I

(8); (H) pHH1508a from DT898 digested with *Xho*I

(9); (I) pHH1508a from P78 digested with *Xho*I

(10); (J) pHH1457-b digested with *Xba*I

(11); (K) R478 digested with *Xba*I

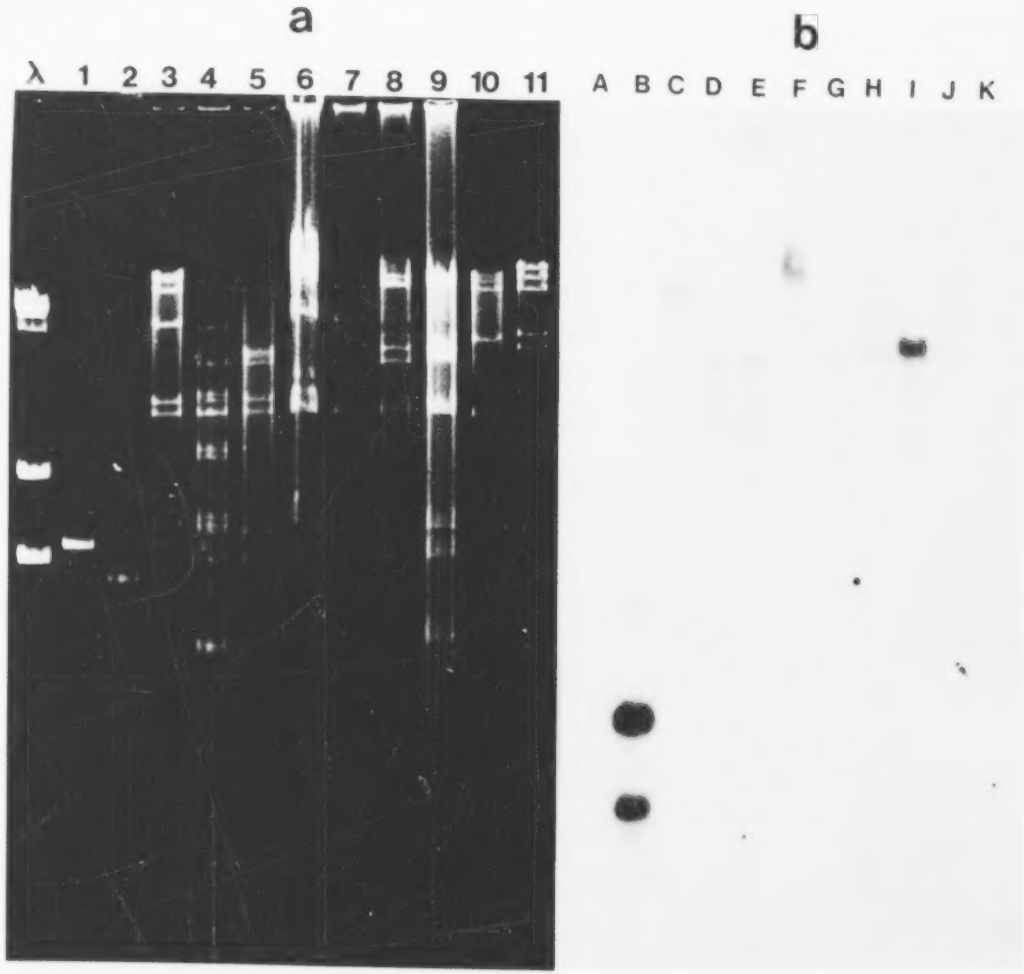

a
λ 1 2 3 4 5 6 7 8 9 10 11
b
A B C D E F G H I J K

FIG. 6 Southern blot hybridization of *Bam*HI-digested MIP233 and *Xho*I-digested pHH1508a using MIP233 Te^r determinant (6.3-kb fragment from MB7H5 cut with *Hin*dIII) as a probe. Lanes 1 through 3 in (a) show ethidium bromide-stained gel and lanes A through C in (b) show the corresponding hybridization patterns.

Lane (1); (A) *Bam*HI-digested MIP233

(2); (B) pHH1508a from DT975 digested with *Xho*I

(3); (C) pHH1508a from DT898 digested with *Xho*I

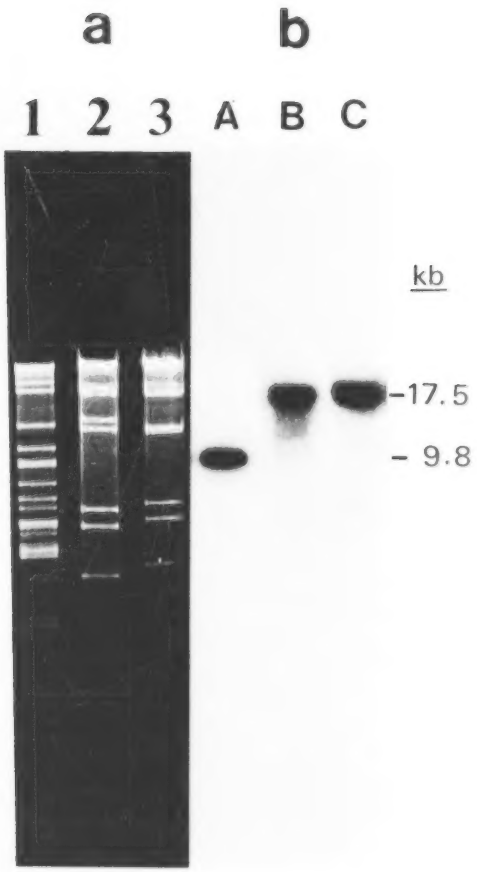

a
b
1 2 3 A B C
kb
17.5
9.8

FIG. 7 Southern blot hybridization of Te^r determinants using the IncHI2 R478 Te^r determinant as a probe. Lanes 1 through 9 in (**a**) show ethidium bromide-stained gel and lanes a through i in (**b**) show the corresponding hybridization patterns. Lane (λ) is λ phage DNA digested with *Hin*dIII.

Lane (1); (a) R478 digested with *Xba*I

(2); (b) pKFW6b digested with *EcoR*I /*Sal*I.

(3); (c) pMJ606 digested with *Pst*I / *Bgl*II

(4); (d) pDAK2 digestedwith *Hin*dIII

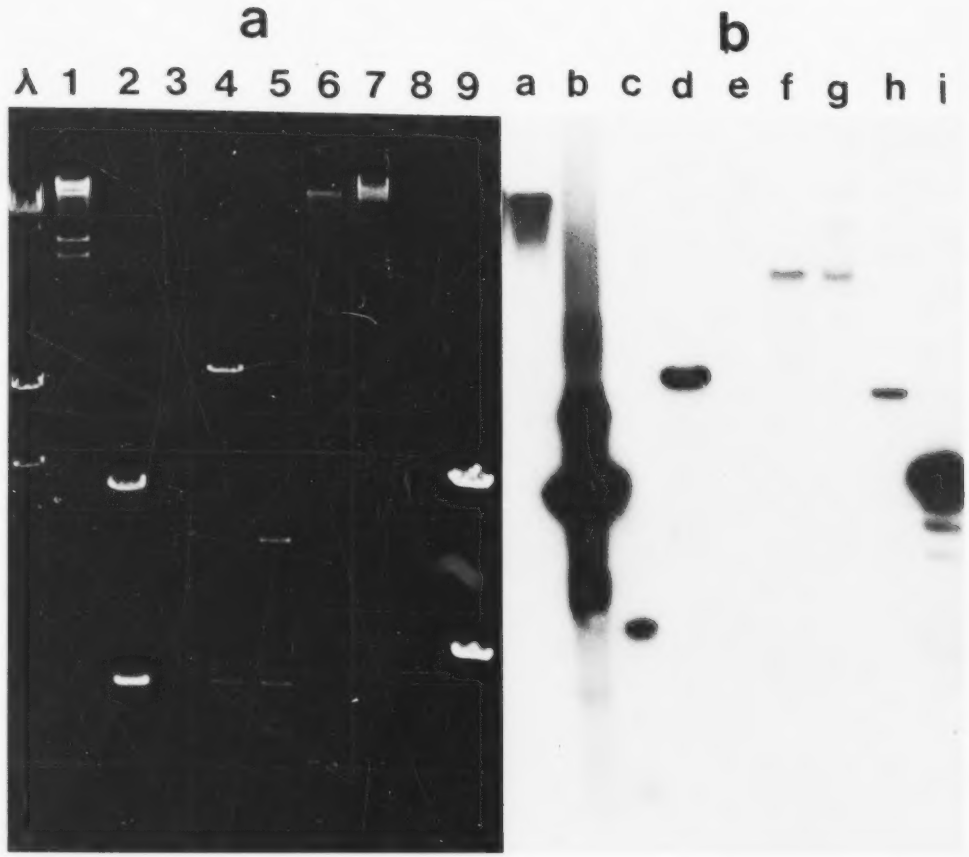
(5); (e) pDT1364 digested with *Xho*I / *Sal*I

(6); (f) pHH1508a from DT898 digested with *Xho*I

(7); (g) pHH1508a from DT975 digested with *Xho*I

(8); (h) MIP233 digested with *Hin*dIII

(9); (i) MB7H5 digestedwith *Hin*dIII


a
b
λ 1 2 3 4 5 6 7 8 9
a b c d e f g h i

Part B. Identification of the Te^r Determinant of pDT1364 (*tehAB*) on the *E. coli* Chromosome

3.6. PCR Amplification of *tehA* and *tehB* genes from a Variety of Different *Escherichia coli* Strains

PCR amplification of 23S rRNA gene, *tehA*, *tehB* and *tehAB* genes were carried out as described in Section 2.16 of materials and methods. The PCR amplified products of 23S rRNA gene, *tehA*, *tehB* and *tehAB* genes from DNA of *E. coli* J53-1, pUC8 and pDT1364 are shown in FIG. 8. PCR reactions which contained no DNA template (lane 4) or inappropriate combinations of DNA template (DNA of pDT1364 or pUC8) (lane 2 and 3 in panel A) with primers (the 23S primers) did not amplify any DNA fragments. This suggests that neither contamination nor nonspecific amplification occurred in the PCR. As expected, the PCR containing the DNA template of *E. coli* J53-1 and 23S primers produced a portion of 23S rRNA gene of 603bp. Amplification reactions from the purified pDT1364 DNA using different primer-pairs, including RTUPCR7-RTUPCR8 (*tehA*), RTUPCR9-RTUPCR10 (*tehB*), and RTUPCR7-RTUPCR10 (*tehAB*) generated DNA fragments of the expected sizes including 1018bp, 621bp and 1610bp, respectively. No DNA fragments were generated from the pUC8 DNA template using the same three primer-pairs indicating that pUC8 did not contain any sequence homologous to Te^r genes of pDT1364 and that the Te^r genes did not originate from the vector pUC8 used to construct pDT1364. Two DNA fragments which have the same sizes as the amplified products of *tehA* and *tehB* from pDT1364 DNA were amplified from *E. coli* J53-1 in two different PCR reactions respectively. The combined fragment, *tehAB*, which is composed of *tehA* and *tehB* was also amplified from *E. coli* J53-1. All the three fragments were shown to have comparable intensity to those amplified from pDT1364. Therefore, both genes *tehA* and *tehB* are not only present in *E. coli* J53-1 but also are arranged in the same way as they are in pDT1364, that is as neighbouring genes.

FIG. 8. PCR amplified products of 23S rRNA gene, *tehA*, *tehB* and *tehAB* genes from DNA of *E. coli* J53-1, pUC8 and pDT1364 subjected to electrophoresis on a 1.1% agarose gel and visualized by ethidium bromide staining. A volume of 10 µl of each mixture was loaded. (A), (B), (C) and (D) represent amplification of 23S rRNA gene, *tehB*, *tehA* and *tehAB*, respectively.

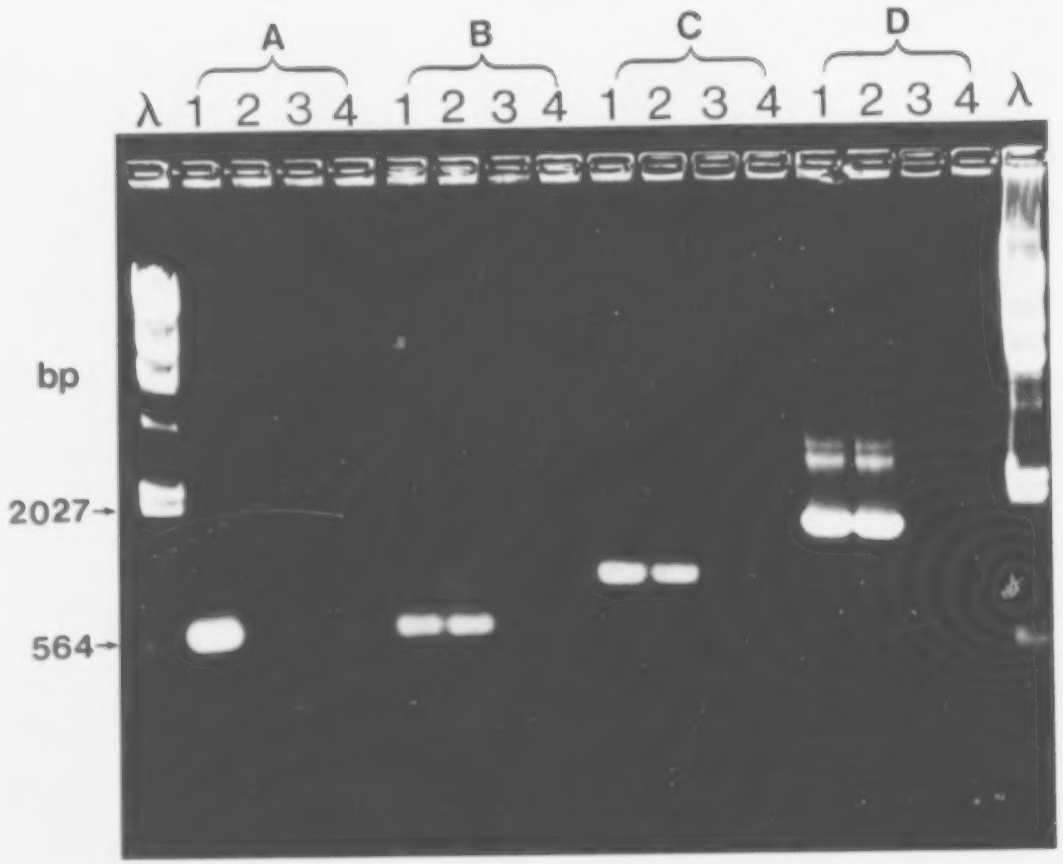
Lane (λ) λ phage DNA digested with *Hind*III

(1) *E. coli* J53-1

(2) pDT1364

(3) pUC8

(4) PCR reaction without any DNA template


A
B
C
D
λ 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 λ
bp
2027
564

These results suggest that the insert present in pDT1364 has originated from the *E. coli* K-12 chromosome.

Another nine *E. coli* K-12 strains including W3110, JC1569, RG192, JM105, HB101, JE2571-4, BB4, DH5α and JM83 were tested for the possible presence of *tehA* and *tehB* genes by PCR. DNA fragments corresponding to 23S rRNA gene, *tehA*, *tehB* and *tehAB* were each amplified from the DNA templates of these 9 *E. coli* K-12 strains. Thus, all 9 *E. coli* K-12 strains tested contained the Te^r region of pDT1364. Other *E. coli* strains including *E. coli* B/R, C and W were also tested for the possible presence of Te^r genes of pDT1364 by PCR. PCRs using DNA templates of *E. coli* B/R, C and W and using the four different primer-pairs including 23S primers, RTUPCR7-RTUPCR8, RTUPCR9-RTUPCR10 and RTUPCR7-RTUPCR10 generated DNA fragments of 603bp, 1018bp, 621bp and 1610bp, respectively. Therefore *tehA* and *tehB* genes are also present in *E. coli* B/R, C and W. The results of PCR amplification of the genes of *tehA* and *tehB* from all the *E. coli* strains tested are shown in FIG. 9 and are summarized in Table 9.

FIG. 9 Agarose gel electrophoresis of PCR amplification of 23S rRNA gene, *tehA* and *tehB* genes from DNA of different *Escherichia coli* strains. A volume of 10 µl of each mixture was loaded onto a 1.1% agarose gel and visualized by ethidium bromide staining.

(1) DNA amplification of *tehA* to give DNA fragment of 1018bp

(2) DNA amplification of *tehB* to give DNA fragment of 621bp

(3) DNA amplification of *tehAB* to give DNA fragment of 1610bp

(4) DNA amplification of 23S rRNA gene to give DNA fragment of 603bp

Lane (λ) λ phage DNA digested with *Hind*III

(A) *Escherichia coli* W3110

(B) Plasmid pDT1364 DNA

(C) No DNA template

(D) J53-1

(E) JC1569

(F) RG192

(G) JM105

(H) HB101

(I) JE2571-4

(J) BB4

(K) DH5α

(L) *E. coli* B/R

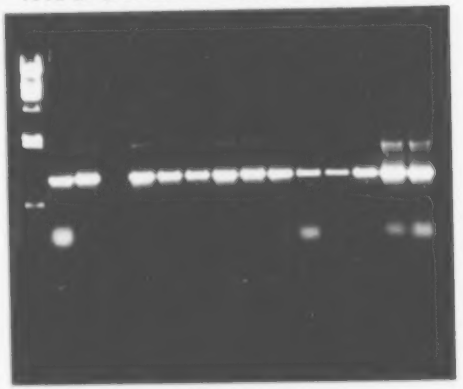
(M) *E. coli* C

(N) *E. coli* W

(O) JM83

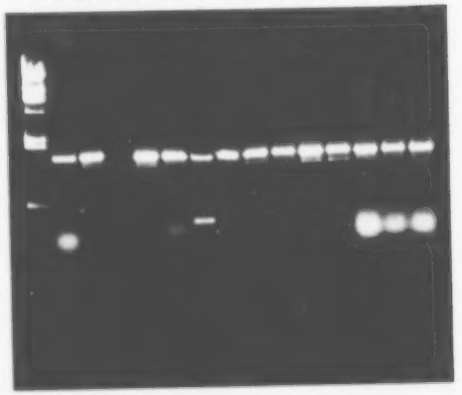
λ A B C D E F G H I J K L M N O

1

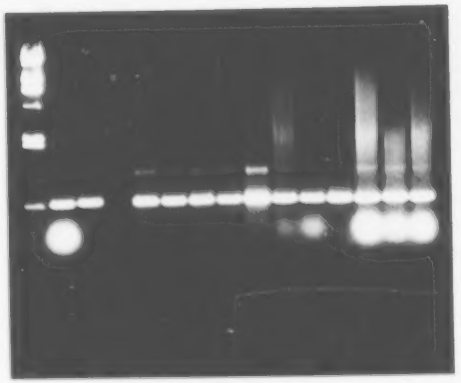


λ A B C D E F G H I J K L M N O

3



2



4

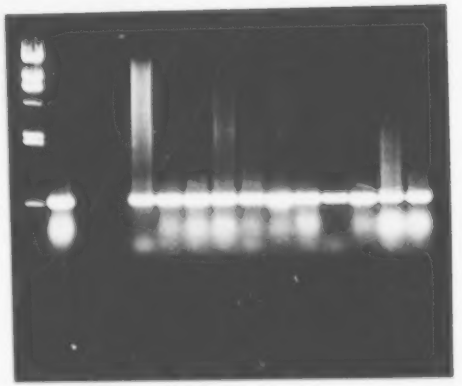


Table 9. PCR Amplification of *teh*A and *teh*B Genes from Different *E. coli* Strains

DNA Template	PCR Product			
	23S rRNA	*teh*A	*teh*B	*teh*A B
W3110	+	+	+	+
pDT1364	-	+	+	+
J53-1	+	+	+	+
JC1569	+	+	+	+
RG192	+	+	+	+
JM105	+	+	+	+
HB101	+	+	+	+
JE2571-4	+	+	+	+
BB4	+	+	+	+
DH5α	+	+	+	+
E. coli B/R	+	+	+	+
E. coli C	+	+	+	+
E. coli W	+	+	+	+
JM83	+	+	+	+

3.7. Hybridization of *tehA* and *tehB* Genes to the Kohara Miniset

According to the result obtained above, all of the *E. coli* strains examined were found to contain the Te^r genes of pDT1364. To map the genes on the *E. coli* K-12 chromosome, the DNA fragments of *tehA* and *tehB* genes were amplified from the plasmid pDT1364 DNA in two PCR reactions using primer-pairs, RTUPCR7-RTUPCR8 and RTUPCR9-RTUPCR10, respectively. They were radiolablled with [α-^{32}P] dCTP by using a commercial Nick Translation System (Gibco. BRL) kit and were used as hybridization probes for localization of *tehA* and *tehB* genes on the Kohara miniset library. The Kohara miniset consists of 476 lambda phage clones covering the entire *E. coli* genome with ample overlapping. It is available in an ordered array supplied either on six 96-well microtiter plates or on a single sheet of hybridization membrane. The order of the lambda clones arranged in the membrane is shown in FIG. 10. The *E. coli* genome map positions were then obtained by reference to Table 10. When the membrane was probed with radiolabelled *tehA* fragment, two clones, #270 (24D6) and #271 (1A6) showed strong hybridization signals, with a stronger signal from #271 (shown in FIG. 11A). When the membrane was probed with the radiolablled *tehB* fragment, two clones, #271 (1A6) and #272 (10D6) gave equally strong signals (shown in FIG. 11B). No other weaker signals were observed in the two autoradiograms. The hybridized clones #270~272 are three consecutive and overlapping clones in the Kohara miniset library with locations of #270 at kb 1499-1508.5, of #271 at kb 1500-1515.5 and of #272 at kb 1509.5-1525.5. The clones #270 and #272 do not overlap and they are separated by a space of about 1kb between their ends. Because *tehA* and *tehB* are two neighbouring genes in the same operon of about 1.6kb in length, they were mapped at a position between kb 1508 and kb 1510 (32.3 min coordinate) covering the spacing region between clones #270 and #272.

FIG. 10. Positions of the "mini-set" clones on the Gene Mapping Membrane.
The DNAs of 476 "mini-set" clones are placed on the membrane at corresponding positions.

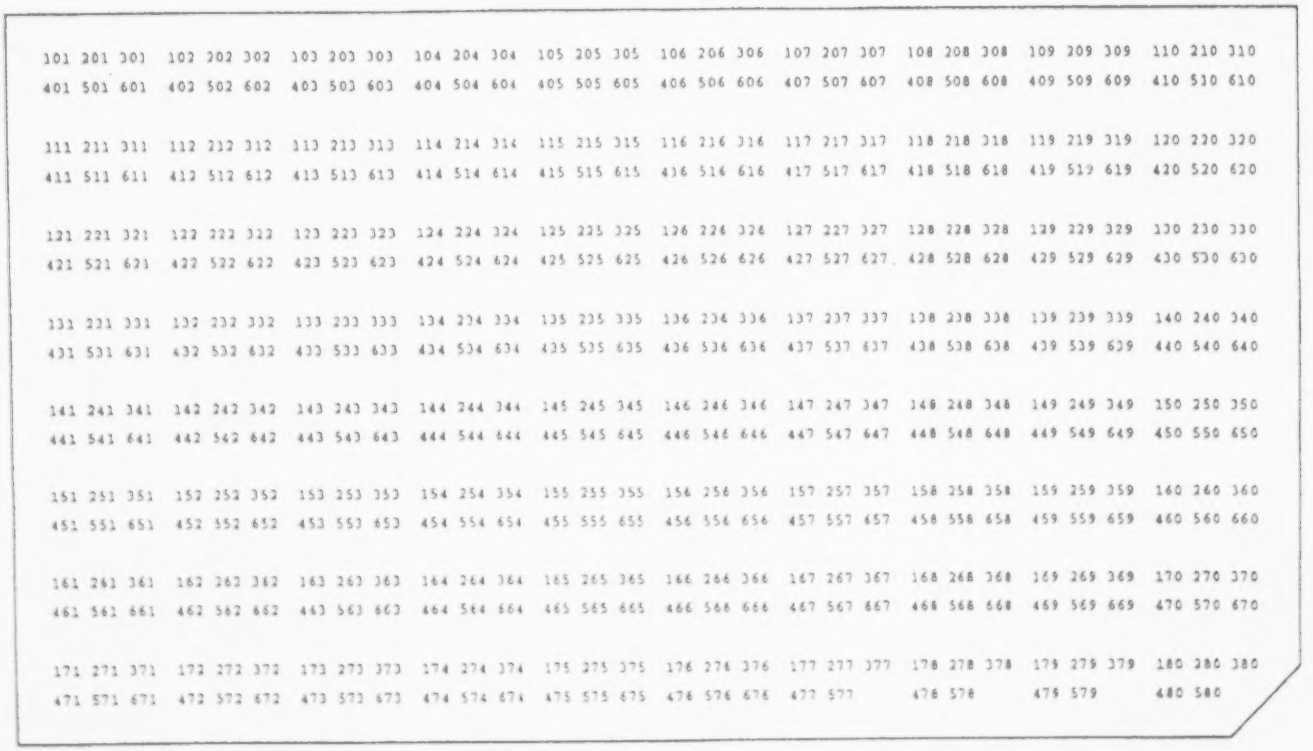


Table 10. Correlation between "mini-set" serial numbers and clone names of Kohara *et al.* (1987).

Serial No. 101-180

	1	2	3	4	5	6	7	8	9	10
100	9E4	6H3	22B12	2F7	4A3	8D2	5H5	8H11	6C1	6F3
110	15B8	4D12	4E11	17C11	11C5	15A7	9H2	4E4	21C8	12D5
120	E2B8	6F5	10F9	8G3	7D5	4A11	8F9	8G4	5A5	3C7
130	21C10	5E5	3E3	9G4	6E6	1F6	9F1	10A6	7H10	8F10
140	6E2	1A10	6A12	9G9	4F3	19F6	2H5	3B6	7E2	19B7
150	8C4	12H5	4B10	7C12	8E4	9E5	6E7	2C4	8F11	23E10
160	12A1	2F5	21A9S	2D12	25A7	4A5	3G5	1G6	15D7	3A2
170	16A8	3A6	11A10	3G6	25C10S	7E10	18F11	10G5	4H1	10F3

Serial No. 201-280

	1	2	3	4	5	6	7	8	9	10
200	4H7	3D4	24F9	1B4	10A8	4C6	3E5	1E2	3H12	5F4
210	14H6	1E5	1H1	1F10	E6H3	13E3	6D12	E1D1	11F5	7F6
220	9E9	E2E5	9B10	4H9	4H11	2F1	5A12	7B5	10E11	9G7
230	1C7	1H7	E4H10S	E3G11	14C1	E9G1	15A8	E4C2	7F9	20E6
240	3E11	4D1	2A3	11G8	7C10	12A3	4D10	13H6	12H9	4D8
250	3D5	14C4	4F1	13F9	18B6	E14F6	1C2	2D3	3H8	6B4
260	3G3	4A6	2C3	4E7	10E6	5F1	1A7	7F12	3C9	24D6
270	1A6	10D6	5F11	8B7	6D4	1G4	1G9	9B8	10C7	22E3

Serial No. 301-380

	1	2	3	4	5	6	7	8	9	10
300	6E8	2A9	5F9	1F9	2H2	2B2	22D11	12E2	7B6	6F6
310	E3F2	11H12	6A3	6A5	9A1	20B5	6F11	4H6	13H4	15G10
320	8G8	20F5	20H8	8A4	19B6	4F7	5E12	4B8	12H7	3E12
330	9F2	7F2	6D1	12B3	15D5	19H3	20H4	12C7	7F11	18A9
340	16B12	4B9	3B3	25D8	9G6	7D4	9B2	3B12	2G4	6D9
350	21H10	1G1	10A9	7A3	2E1	3G4	1H4	10H4	8F4	3E4
360	2B4	17A4	1E1	2B1	7F1	7H12	5H11	4F2	12C12	1F2
370	20F6	19D1	22A6	5A1	4F12	E13A5	20E7	22C4	4B4	5H12

Table 10. Continued.

Serial No. 401-480

	1	2	3	4	5	6	7	8	9	10
400	9F11	4C8	E9B9	9C2	E9C9	9D2	12F12	2G11	2C9	6F7
410	3A11	6B9	7G9	10D3	12H4	4G10	3E7	12G2	5F7	E8E3
420	4E10	7A8	4C11	5A8	10H6	5A11	8E3	2D5	7F8	5E10
430	6F10	8E12	6H2	7G4	4A12	3F10	21D7	E8F2	22D7	24G1
440	4C9	2D4	12G12	10H9	8G10	23B7	9G10	1B2	12F2	25D2
450	1B5	6C8	9A10	1B3	8B9	10B5	8C5	9A12	10B6	3G11
460	5A10	8H3	12H3	15G6	10D8	6A1	5B4	22H4	5E11	10B4
470	1A2	6C5	1H10	23G4S	12C6	3A9	3D11	1G7	21H2	3B2

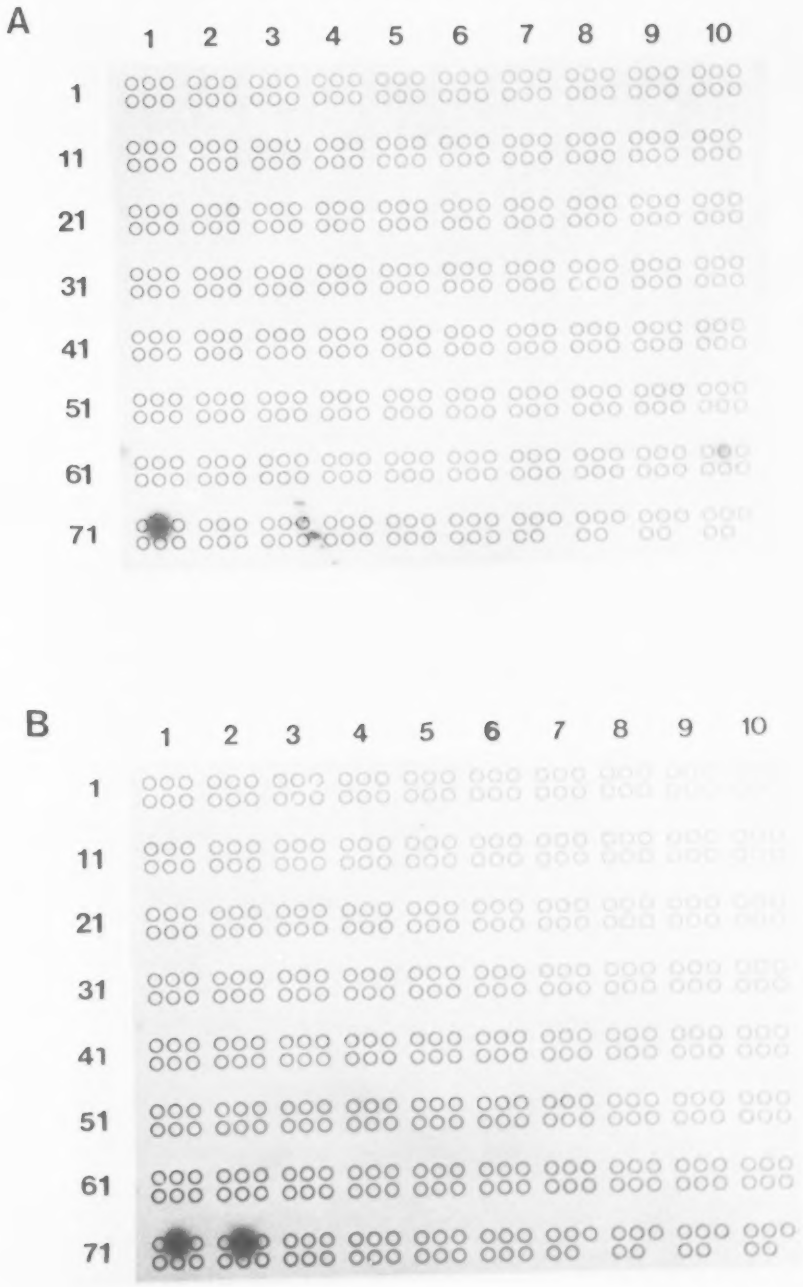
Serial No. 501-580

	1	2	3	4	5	6	7	8	9	10
500	8A5	2F9	2B7	5C10	6H4	17B2	6B12	9F9	19F2	17E4
510	4A1	15B3	21D1	8F8	6B5	20F4	16C4	13A9	E4G11S	18H7
520	14F11	7E3	3G10	8D6	7C8	1E3	3G9	21D3	6G3	6G9
530	3C5	9B9	7B7	E11C11	4G11	8H10	6D8	12E3	4H12	8B10
540	4B6	7G8	6H5	6H9	9H1	10F1	8D12	10H11	9B11	16G1
550	1C10	7G3	12G1	10F4	7B1	2A8	2E6	15D10	4F4	2A1
560	7A1	5E3	2A2	3F11	3C10	3D1	8E8	7C4	1D11	12D11
570	7F3	2A6	4D2	16B11	5D2	17G2	2C10	9B3	7C3	10C8

Serial No. 601-676

	1	2	3	4	5	6	7	8	9	10
600	6F2	9F6	1D1	9H8	25B12S	9G3	4D9	12E4	6A4	10D10
610	5F2	7H7	1B6	5B10	10F5	16A5	E5B4	E4E4	E3C10	2F6
620	1C9	4D4	6F9	15C3	4C5	3F8	9H3	E1H9	18C4	5F12
630	6G10	4E5	7A4	1F8	12B4	1E4	12D1	3F12	10G7	1A11
640	12A6	10H5	12H2	2C12	8H1	5H2	21H11	5G7	9B1	1D4
650	3H6	3A1	6G4	1G10	7E9	5B5	E1H3	E1F5	5C4	E4D8
660	16H7S	7G7	9H9	19H10	3F1	1D6	5G8	8H8	12G5	10H12
670	7C1	8D1	5C1	15C9	12A4	15A5				

FIG. 11. Hybridization pattern of ^{32}P-lablled *tehA* and *tehB* probes on the *E. coli* Gene Mapping Membrane. The membrane was probed with ^{32}P-lablled *tehA* (A) and *tehB* (B) DNA fragments. Two positive hybridization signals were seen using each probe. With the *tehA* probe, positive signals were assigned to clones #270 (24D6) and #271 (1A6). With the *tehB* probe, positive signals were assigned to clones #271 (1A6) and #272 (10D6).


A
1 2 3 4 5 6 7 8 9 10
1
11
21
31
41
51
61
71
B
1 2 3 4 5 6 7 8 9 10
1
11
21
31
41
51
61
71

To confirm this location, dot blot hybridization of *tehA* and *tehB* to the three positive phage clones #270-272 was performed. Two other clones #170 and #171 were randomly chosen from the Kohara miniset and were used as negative controls. The DNA of these clones was isolated and purified as described in section 2.15. Two duplicate dot blots (A and B, dots: 1, #170; 2, #171; 3, #270; 4, #271; 5, #272) were made from the DNA of these five samples. The PCR amplified fragments of *tehA* and *tehB* genes from pDT1364 were radiolabelled and used as hybridization probes. As shown in FIG. 12, DNA of #270 and #271 hybridized to *tehA*; DNA of #271 and #272 hybridized to *tehB*; whereas no hybridization was observed between either of the probes and the negative control clones. These results verified that the three positive lambda clones identified by hybridization to the Kohara miniset membrane were the correct clones containing *tehA* and *tehB* genes.

FIG. 12 Autoradiogram showing hybridization of ^{32}P-labelled *tehA* and *tehB* genes from pDT1364 with DNA of selected Kohara miniset clones. Blot A was probed with *tehA*; blot B was probed with *tehB*. Dots: 1, #170; 2, #171; 3, #270; 4, #271; 5, #272.

A

1 2 3 4 5

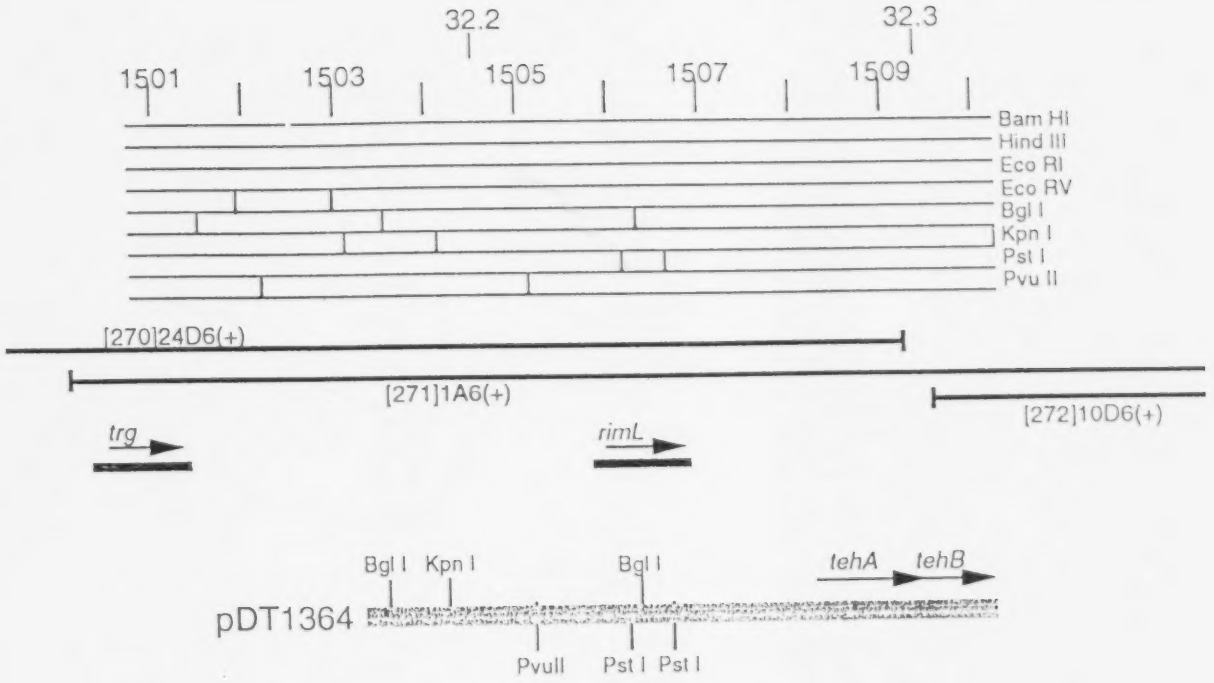
B

1 2 3 4 5

3.8 Alignment of the Restriction Map of the pDT1364 Insert with the *E.coli* Physical Map

The *E. coli* physical map was constructed by Kohara *et al* (1987) from *Sau*3AI partial digests of *E. coli* W3110 DNA using eight six-base-recognizing endonucleases, namely *Bam*HI, *Hind*III, *EcoR*I, *EcoR*V, *Bgl*I, *Kpn*I, *Pst*I and *Pvu*II. It has been integrated with a genetic map by Rudd *et al.* (1992, version 6), which contains more information and is more accurate. The restriction map of the 6.8-kb insert on pDT1364 was also constructed using the same eight restriction enzymes. As shown in FIG. 13, the restriction map of the 6.8-kb insert on pDT1364 could be aligned completely with that of the *E. coli* chromosome at a region from kb 1503 to 1510. This suggests that the 6.8-kb insert had originated from the *E. coli* K-12 chromosome due to contamination by chromosomal DNA in the plasmid preparation of pHH1508a.

FIG. 13. Location of the *tehA* and *tehB* genes on the *E. coli* chromosome. The scales at the top refer to positions in minutes (upper) and in kilobases (lower). The restriction map of the 1501- to 1510-kb region of the *E. coli* chromosome is shown in the format of Kohara *et al* (1987), with the restriction map of the insert of pDT1364 (in shaded box) also in the same format (at the bottom). The locations of the inserts of the relevant lambda clones are shown in bold lines. The locations of the genes in this region and the directions of transcription are indicated by arrows.


32.2
32.3
1501
1503
1505
1507
1509
Bam Hi
Hind III
Eco RI
Eco RV
Bgl I
Kpn I
Pst I
Pvu II
[270]24D6(+)
[271]1A6(+)
[272]10D6(+)
trg
rimL
Bgl I
Kpn I
Bgl I
tehA
tehB
pDT1364
PvuII
Pst I
Pst I

3.9. Searching the *E. coli* Deletion Mutants for the *tehA* and *tehB* Genes by PCR Amplification and Dot Blot Hybridization

The *tehA* and *tehB* genes of pDT1364 had been identified and located within the *E. coli* chromosome at kb1508 and 1510 (32.3 min coordinate) by PCR and by comparison with the restriction maps. To provide further proof, two *E. coli* mutants PK1427 (Henson *et al.*, 1984) and PK1439 (P. L. Kuempel, personel communication) with deletions in the terminus region were examined for the presence of the *tehA* and *tehB* genes. PK1439 contains a 140-kb deletion across the region where the *tehA* and *tehB* genes are located. PK1427 contained a 70-kb deletion which did not involve the region of the *tehA* and *tehB* genes. The genomic DNA was isolated from the two strains and was subjected to PCR amplification using primer-pairs, RTUPCR7-RTUPCR8 and RTUPCR9-RTUPCR10. As shown in FIG. 14, the DNA fragments of the *tehA* and *tehB* genes were amplified from PK1427 but were not generated from PK1439. Thus PK1427 still contained the *tehA* and *tehB* genes whereas PK1439 did not. To confirm these results, DNA dit-blots of these two strains were subjected to hybridization with radiolabelled PCR amplified *tehA* and *tehB* fragments derived from pDT1364. Two wild-type *E. coli* strains W3110 and J53 were used as positive controls. Lambda phage was used as a negative control. As shown in FIG. 15, the two positive control samples W3110 and J53 as well as PK1427 hybridized to both probes; whereas the negative control sample, λ phage and PK1439 did not hybridize to either of the two probes.

FIG. 14 Agarose gel electrophoresis of PCR amplification of 23S rRNA gene, *tehA* and *tehB* genes from DNA of different *Escherichia coli* deletion mutants. A volume of 10 µl of each mixture was loaded onto a 1.1% agarose gel.

(A) DNA amplification of 23S rRNA gene

(B) DNA amplification of *tehB*

(C) DNA amplification of *tehA*

(D) DNA amplification of *tehAB*

Lane

(λ) λ DNA digested with *Hin*dIII

(1) W3110

(2) PK1427

(3) PK1439

(4) No DNA template control

A
B
C
D
λ 1 2 3 4 λ 1 2 3 4 λ 1 2 3 4 λ 1 2 3 4
bp
2027
564

FIG. 15 Autoradiogram of dot blot hybridization of the *E. coli* deletion mutants with ^{32}P-labelled *tehA* and *tehB* genes of pDT1364. Two wild-type *E. coli* strains i.e. W3110 and J53 were used as positive controls. Lambda phage was used as a negative control. (A) was probed with *tehA*; (B) was probed with *tehB*.

Dots:

(1) W3110

(2) J53

(3) λ phage

(4) PK1439

(5) PK1427

A

1 2 3 4 5

B

1 2 3 4 5

3.10. Dot Blot Hybridization of *tehA* and *tehB* Probes to DNA from Various *E. coli* Strains

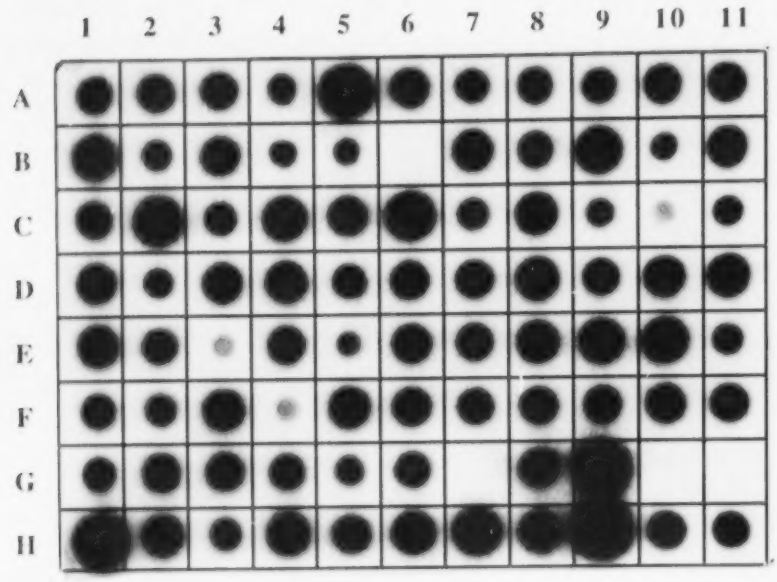
A set of 72 natural isolates of *E. coli* (the ECOR Collection) from a variety of hosts and geographical locations has been characterized and reflects the genotypic diversity in the species (Ochman and Selander, 1984). In order to define the prevalence of *tehA* and *tehB* genes in *E. coli*, the 72 standard reference strains in the ECOR Collection as well as 12 *E. coli* laboratory strains were tested for the possible presence of *tehA* and *tehB* genes by dot blot hybridization of *tehA* and *tehB* probes generated by PCR. Lambda phage and PK1439 were used as negative controls, whereas the purified plasmid pDT1364 was used as a positive control. As shown in FIG. 16, the purified pDT1364 hybridized to both probes; whereas the negative controls λ phage and PK1439 did not hybridize to either of the probes. With the *tehB* probe (B), no hybridization signal was observed from ECOR 9, ECOR 12, ECOR 17 or ECOR 30 whereas the rest hybridized. Another blot was made containing more DNAs of ECOR 9, ECOR 12, ECOR 17 and ECOR 30 and then was probed with the *tehA* probe. As shown in (A), all the *E. coli* strains tested except ECOR 17 hybridized.

To confirm the negative results from these strains, PCR reactions were carried out using primers for *tehA*, *tehB* and 23S rRNA genes. The results (FIG. 17) demonstrated that Ecor 17 lacked both *tehA* and *tehB* whereas Ecor 9, Ecor 12 and Ecor 30 contained both genes.

FIG. 16. Autoradiogram of dot blot hybridization of the ECOR collection and 12 labratory strains with ^{32}P-labelled *tehA* and *tehB* probes generated by PCR. Lambda phage, *H. influenzae* (=H. inf.) and PK1439 were used as negative controls, whereas the purified pDT1364 was used as a positive control. The DNA samples are arranged as follows:

ECOR 1	ECOR 2	ECOR 3	ECOR 4	ECOR 5	ECOR 6	ECOR 7	ECOR 8	ECOR 9	ECOR 10	ECOR 11
ECOR 12	ECOR 13	ECOR 14	ECOR 15	ECOR 16	ECOR 17	ECOR 18	ECOR 19	ECOR 20	ECOR 21	ECOR 22
ECOR 23	ECOR 24	ECOR 25	ECOR 26	ECOR 27	ECOR 28	ECOR 29	ECOR 30	ECOR 31	ECOR 32	ECOR 33
ECOR 34	ECOR 35	ECOR 36	ECOR 37	ECOR 38	ECOR 39	ECOR 40	ECOR 41	ECOR 42	ECOR 43	ECOR 44
ECOR 45	ECOR 46	ECOR 47	ECOR 48	ECOR 38B	ECOR 50	ECOR 51	ECOR 52	ECOR 53	ECOR 54	ECOR 55
ECOR 56	ECOR 57	ECOR 58	ECOR 59	ECOR 60	ECOR 61	ECOR 62	ECOR 63	ECOR 64	ECOR 65	ECOR 66
ECOR 67	ECOR 68	ECOR 69	ECOR 70	ECOR 71	ECOR 72	λ phage	W3110	pDT 1364	PK 1439	H. inf.
J53-1	JC1569	RG192	JM105	HB101	JE 2571-4	BB4	DH5α	B/R	C	W

A



B

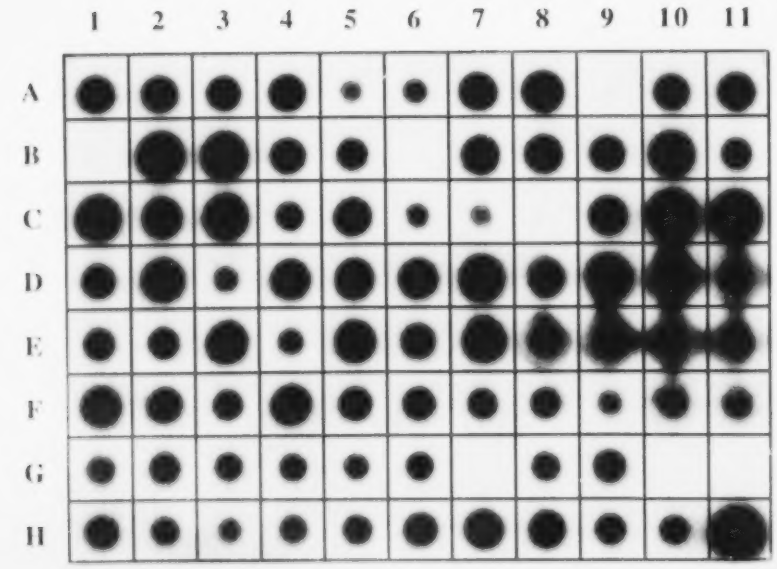


FIG. 17 Agarose gel electrophoresis of PCR amplification of 23S rRNA gene, *tehA* and *tehB* genes from DNA of Ecor 9, Ecor 12, Ecor 17 and Ecor 30. The strain *E. coli* W3110 was used as a positive control.

(A) DNA amplification of 23S rRNA gene

(B) DNA amplification of *tehA*

(C) DNA amplification of *tehB*

Lane (1) *E. coli* W3110

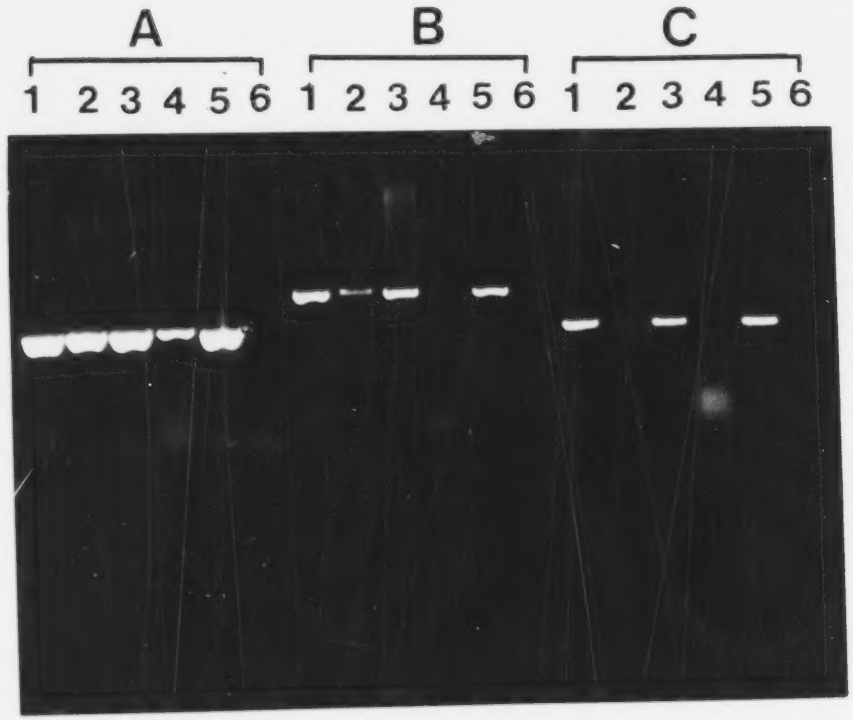
(2) Ecor 9

(3) Ecor 12

(4) Ecor 17

(5) Ecor 30

(6) No DNA template control


A
B
C
1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6

3.11. Searching a Variety of Other Bacteria for the *tehA* and *tehB* Genes by PCR Amplification and Dot Blot Hybridization

The DNA from 12 other bacterial strains including *Salmonella typhimurium* LT2, *Shigella sonnei*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Arizona* spp., *Citrobacter*, *Proteus vulgaris*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, *Campylobacter jejuni*, *Corynebacterium diphtheriae* and *Staphylococcus aureus* were tested for the presence of *tehA* and *tehB* genes by PCR amplification. The fragment of 23S rRNA gene was amplified from all these bacteria by PCR using the 23S primers. No DNA fragments of any length were generated from PCR reactions using the primers for *tehA* and *tehB* genes. The results are shown in FIG. 18. and are summarized in Table 11. None of the species tested contained the Te^r genes from pDT1364.

To confirm these results, dot-blot hybridization of the DNAs from these bacteria to the ^{32}P-radiolabelled *tehA* and *tehB* fragments of pDT1364 was carried out. The two *E.coli* strains W3110 and J53 were used as positive controls for hybridization. The Te^r region-deleted *E. coli* strain PK1439 and lambda were employed as negative controls. The blot was probed with ^{32}P-radiolabeled PCR amplified *tehA* and *tehB* fragments from pDT1364. As shown in FIG. 19, no hybridization signal was observed from any of these bacteria (E to P) and the two negative control samples (C and D) whereas the two *E.coli* strains W3110 (A) and J53 (B) hybridized with both the probes.

FIG. 18 Agarose gel electrophoresis of PCR amplification of 23S rRNA gene, *tehA* and *tehB* genes from DNA of different bacterial strains. A volume of 10 µl of each mixture was loaded onto a 1.1% agarose gel. (1) DNA amplification of *tehA*; (2) DNA amplification of *tehB*; (3) DNA amplification of 23S rRNA gene. Lane marked (λ) is λ DNA digested with *Hind*III. DNA templates used for amplication were used as follows:

Lane (A) *Escherichia coli* W3110

(B) Plasmid pDT1364 DNA

(C) No DNA template

(D) *Salmonella typhimurium* LT2

(E) *Shigella sonnei*

(F) *Klebsiella pneumoniae*

(G) *Enterobacter cloacae*

(H) *Arizona* species

(I) *Citrobacter* species

(J) *Proteus vulgaris*

(K) *Haemophilus influenzae*

(L) *Pseudomonas aeruginosa*

(M) *Campylobacter jejuni*

(N) *Corynebacterium diphtheriae*

(O) *Staphylococcus aureus*

λ A B C D E F G H I J K L M N O

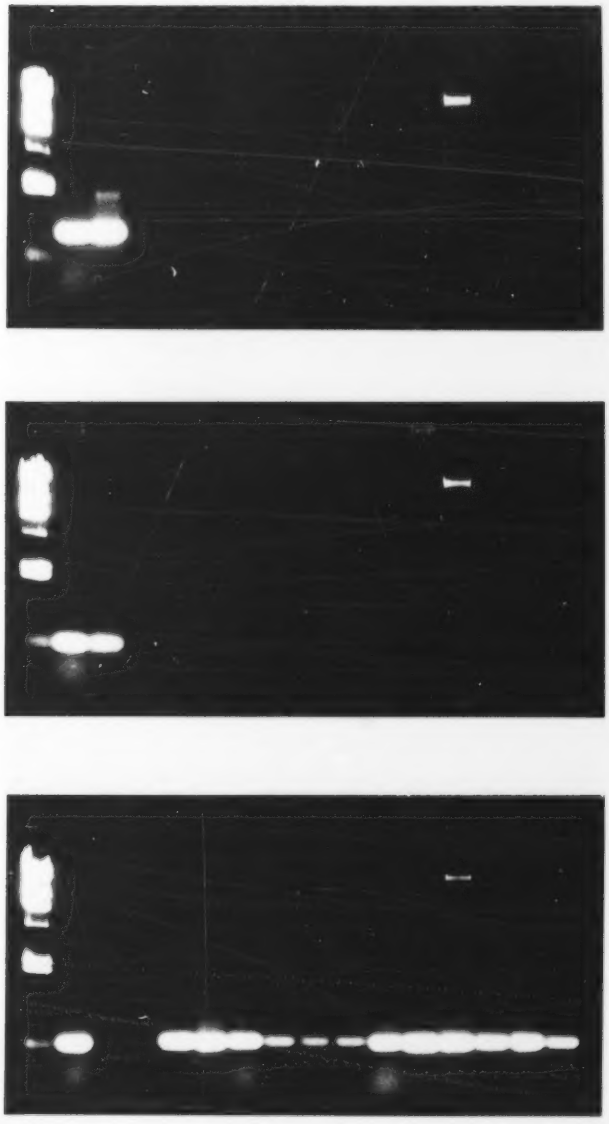


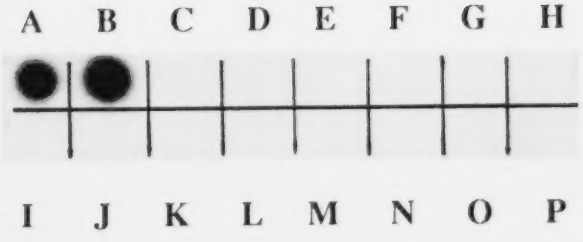
Table 11. PCR Amplification of *tehA* and *tehB* Genes from Different Bacteria

	PCR Product		
DNA Template	**23S rRNA**	***tehA***	***tehB***
Escherichia coli W3110	+	+	+
Plasmid pDT1364 DNA	-	+	+
Salmonella typhimurium LT2	+	-	-
Shigella sonnei	+	-	-
Klebsiella pneumoniae	+	-	-
Enterobacter cloacae	+	-	-
Arizona spp.	+	-	-
Citrobacter	+	-	-
Proteus vulgaris	+	-	-
Haemophilus influenzae	+	-	-
Pseudomonas aeruginosa	+	-	-
Campylobacter jejuni	+	-	-
Corynebacterium diphtheriae	+	-	-
Staphylococcus aureus	+	-	-

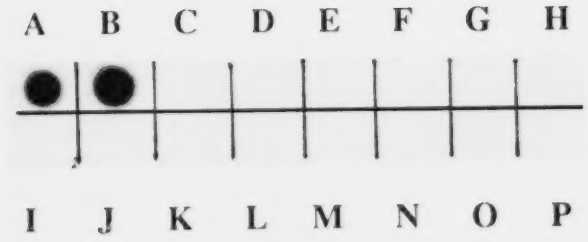
FIG. 19. Autoradiogram showing dot blot hybridization of DNA samples from a variety of different bacterial strains to ^{32}P-labeled *tehA* and *tehB* fragments from pDT1364 by PCR. Duplicate blots (a and b) were probed with ^{32}P-labelled *tehA* and *tehB*, respectively.

Dot
(A) *E.coli* W3110
(B) *E. coli* J53
(C) λ phage
(D) PK1439
(E) *Salmonella typhimurium* LT2
(F) *Shigella sonnei*
(G) *Klebsiella pneumoniae*
(H) *Enterobacter cloacae*
(I) *Arizona* species
(J) *Citrobacter* species
(K) *Proteus vulgaris*
(L) *Haemophilus influenzae*
(M) *Pseudomonas aeruginosa*
(N) *Campylobacter jejuni*
(O) *Corynebacterium diphtheriae*
(P) *Staphylococcus aureus*

a



b



3.12. MICs of Tellurite for Various Plasmid-free *E. coli* Strains

The MICs of K_2TeO_3 for various *E. coli* strains were determined using the agar dilution method described in Section 2.7. The MICs for a variety of *E. coli* laboratory strains carrying chromosomal *tehAB* genes are shown in Table 12. They vary from 0.1 to over 256μg/ml. Most of these strains had MICs of 2 to 8μg/ml. A few strains including JC1569, JM105 and DH5α had very low MICs at 0.4, 0.1 and 0.64μg/ml. However one strain, *E. coli* BB4 had a MIC greater than 256μg/ml.

ECOR 17 was the only strain of the ECOR collection which did not contain either *tehA* or *tehB*. Its MIC was also determined. In spite of the absence of the *tehAB* genes on its chromosome, this strain specified an MIC of 2μg/ml which is very similar to the MICs of most *E. coli* lab strains carrying chromosomal *tehAB* genes (Table 12).

Two *E. coli* deletion mutants PK1427 (Henson *et al.*, 1984) and PK1439 (P. L. Kuempel, personel communication) which contain deletions in the terminus region were tested for the MIC of K_2TeO_3. PK1427 has a 70kb-deletion but contains an intact *tehAB* operon. It specified an MIC of 4μg/ml. Mutant PK1439, containing a 140-kb deletion across the region where the *tehAB* operon is located, specified an MIC (0.2μg/ml) 20 times lower than the MICs of most *E. coli* lab strains carrying chromosomal *tehAB* genes (Table 13).

Table 12. MICs of Tellurite for Various *E. coli* Laboratory Strains Carrying Chromosomal *tehAB* Genes

Strain No.	Strain	Genotype	MIC of K_2TeO_3[a] (μg/ml)
DT384	W3110	*gal* λ⁻	4-8
DT902	J53-1	*pro met*	2
DT121	JC1569	*leuB6 tonA2 supE44 gal-6* λ*hisG1 rfbD1 galP63 argG6 xyl-7 mlA2 metB1 recA1 rpsL104*	0.4
DT192	RG192	Δ*ara leu lac* Rif^r	2
DT1078	JM105	*thi rpsL endA sbcB15 hsdR4* Δ*(lac-proAB)* [F' *traD36 proAB* lacI^{q}Z ΔM15]	0.1
DT1156	HB101	*pro leu thi lacY hsdR end recA rpsL20 ara-14 galK2 xyl-5 mtl-1 supE44* Str^r	1-2
DT1325	JE2571-4	Pil⁻ Fla⁻, spectinomycin resistant	4
DT2544	BB4	*supF58 supE44 hsdR514 galK2 galT22 trpR55 metB1 tonA* Δ*lac*U169 F' [*proAB*$^+$ *lacI*^{q} *lacZ*ΔM15 Tn*10*(*tet*^{r})]	>256
DT2171	DH5α	*supE44* Δ*lac*U169 (*ϕ80 lacZ* ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	0.64
DT2517	*E. coli* B/R	(ATCC11143) radiation resistant *E. coli* B	4-8
DT176	RG176	*E. coli* C strain "C-1a" nalidixic acid resistant	2-4
DT2545	*E. coli* W	(ATCC9637) wild type	4-8
DT1098	JM83	*ara* Δ*(lac-proAB) rpsL*(Str^r) (*ϕ80 lacZ*ΔM15)	2

[a] Determined in both BHI and L agar plates.

Table 13. MICs of Tellurite for *E. coli tehAB* Deletion Strains Habouring Different Plasmids

Strain	Plasmid	MIC of K_2TeO_3[a] (μg/ml)
ECOR17	None	2
ECOR17	pUC8	2
ECOR17	pDT1555	>256
ECOR17	pMJ606	>256
ECOR17	pDT1364	128
PK1439	None	0.2
PK1439	pUC8	0.2
PK1439	pDT1555	>256
PK1439	pMJ606	>256
PK1439	pDT1364	0.2

[a] Determined in both BHI and L agar plates.

3.13. Complementation of the two *E. coli tehAB* deletion strains

During this study, two *E. coli* strains, PK1439 and ECOR 17, were found to be devoid of the *tehAB* operon by PCR using specific primers for *tehA* and *tehB* genes or by hybridization. In order to complement the chromosomal defect these 2 strains were transformed with plasmid pDT1364, containing the entire *tehAB* operon together with 5 kb of the upstream region (Cohen, *et al.*, 1972). Plasmids pUC8, pDT1555 and pMJ606 were also introduced into the strains as controls. The MICs of K_2TeO_3 for the transformants were then determined in both BHI and L agar as described Section 2.7.

The results are summarized in Table 13. The MICs specified by both ECOR 17 (pUC8) and plasmid-free ECOR 17 were 2 μg/ml. However, the MIC for ECOR 17 (pDT1364) was much higher at 128 μg/ml which is the same as the MIC specified by pDT1364 in *E. coli tehAB*-carrying strains.

The MICs specified by PK1439 (pUC8) and the plasmid-free PK1439 are very low at 0.2 μg/ml. Unlike ECOR 17, after pDT1364 was introduced into PK1439, the MIC. The MIC retained at 0.2 μg/ml. Therefore, tellurite resistance could not be complemented by pDT1364 in PK1439.

4. DISCUSSION

Tellurite (TeO_3^{2-}) is toxic to most microorganisms, particularly gram-negative bacteria (Fleming, 1932; Scala and William, 1963). The tellurite resistance (Te^r) in gram-negative bacteria is usually plasmid-mediated. Plasmids of several incompatibility groups have been shown to mediate resistance to tellurite. In the *Enterobacteriaceae*, most of the tellurite-resistant plasmids belong to the IncHI2, IncHI3, IncHII and IncP groups (Taylor and Summers, 1979; Taylor and Levine, 1980; Bradley *et al.*, 1982; Bradley, 1985). The Te^r determinants from IncHI2 plasmid pMER610, IncPα plasmid RK2 and IncHII plasmid pHH1508a have been extensively studied. These determinants have been cloned, sequenced and expressed in *E. coli*. No apparent homology was observed at either DNA level or amino acid level among these three Te^r determinants (Jobling and Ritchie, 1987, 1988; Walter *et al.*, 1991a; c; Walter, Ph.D. thesis, 1990). The Te^r determinants from plasmids MIP233 (IncHI3), R478 (IncHI2) and pHH1457b (IncHII) have also been cloned (Whelan, Ph.D. thesis. 1992; D. Keane, M.Sc. thesis, 1990), but have not been sequenced. The homology among all these Te^r determinants and their prevalence in plasmid populations have not been determined yet. This study was initially an attempt to determine the incidence of these Te^r determinants among plasmids of different incompatibility groups and to examine the Te^r levels specified by a variety of plasmids.

To examine how much homology was present among the cloned Te^r determinants and to determine the incidence of them among plasmids of different incompatibility groups, both dot-blot and Southern hybridization were performed under conditions of high stringency (50% formamide; 42°C). Dot blot hybridization was carried out first in order to screen many plasmid samples at one time. All the cloned Te^r determinants were ^{32}P-radiolabelled and used as hybridization probes. From the results in this study, the

following conclusions were made: (1) All the IncPα plasmids tested contain the IncPα Te^r determinant and do not contain the other Te^r determinants. The IncPα Te^r determinant is found only within the IncPα plasmids. (2) The IncHI2 (pMER610) and IncHI3 (MIP233) Te^r determinants hybridize with one another and are therefore closely related. These Te^r determinants are present in all the IncHI2 except R476b, IncHI3 and IncHII plasmids as well as in all the unclassified Te^r plasmids. This Te^r locus was identified within the *Xho*I "E" fragment in the IncHII plasmid pHH1508a. (3) A small degree of homology is present between the R478 Te^r determinant and the Te^r determinants of pMER610 and MIP233. (4) None of the Te^r determinants is homologous to the *ars* determinant found in pUM3, which could also confer a low to moderate level of Te^r. (Mobley *et al.*, 1983), which was also found to confer a moderate level of Te^r with a MIC of tellurite at 64μg/ml (Turner *et al.*, 1992).

Earlier studies (Walter, 1990, Ph.D thesis) reported that the plasmid pDT1364 was created by cloning the Te^r region of pHH1508a into the *Sal*I site of pUC8. However, when the Te^r determinant of pDT1364 was used as a probe, it did not consistently hybridize back to pHH1508a plasmid DNA. It appeared to hybridize with pHH1508a DNA isolated from a lab stock strain DT898=[*E. coli* J53-1 (pHH1508a)] but not to plasmid DNA from another strain DT975=[*E. coli* RG2137 (pHH1508a)]. One likely explanation was that plasmid pHH1508a might initially have contained two Te^r determinants and the plasmid present in the stock DT975=[*E. coli* RG2137 (pHH1508a)] might have undergone some DNA rearrangement resulting in loss of the Te^r determinant from pDT1364. To test this hypothesis, pHH1508a was isolated from different stocks including DT898, DT975 and P78 =[*E. coli* J53-1 (pHH1508a)] which was one of the original pHH1508a isolates. The DNA was digested with restriction enzymes *Xba*I and *Xho*I which had been used in the contruction of the pHH1508a restriction map (Yan and Taylor, 1987; Yan, M.Sc. thesis, 1986). The restriction patterns were then compared to

each other and also compared to the one previously obtained by Yan (Ph.D thesis, 1986). As shown in FIG. 3, some changes were noted in the restriction pattern of pHH1508a from DT975, whereas the restriction patterns of the plasmid from DT898 and P78 were still the same as that previously obtained by Yan (Ph.D thesis, 1986). This finding was strong support for the proposal.

However, further proof from Southern hybridization analysis was still needed. If rearrangements of plasmid from DT975 accounted for the lack of hybridization between pHH1508a and the pDT1364 Te^r determinant, the plasmid from DT898 or P78, which did not show any changes, was expected to hybridize with the radiolabelled probe of pDT1364 Te^r determinant in Southern hybridization. When Southern hybridization was undertaken the Te^r determinant of pDT1364 did not hybridize to pHH1508a from stocks of either DT898 or P78. It was shown to hybridize with the *E. coli* J53-1 chromosome. In one experiment the pDT1364 Te^r determinant appeared to hybridize to a 9-kb fragment of pHH1508a obtained by *Xho*I and *Xba*I / *Xho*I digestion. This fragment does not belong to pHH1508a according to the pHH1508a restriction map (Yan and Taylor, 1987; Yan, M.Sc. thesis, 1986). This result indicates that pHH1508a does not contain the Te^r determinant of pDT1364. Thus this hypothesis was ruled out and the previous report on the origin of pDT1364 Te^r determinant from pHH1508a (Walter, Ph.D. thesis, 1990; Walter *et al.*, 1991b) was shown to be incorrect. However rearrangements have occurred in the plasmid from DT975, although the reason and the consequences of these rearrangements are not understood. Therefore the origin of pDT1364 Te^r determinant was still mysterious and needed to be investigated.

The second hypothesis, that the insert present in pDT1364 might have originated from *E. coli* K12 chromosome, was based on the observed hybridization of pDT1364 Te^r determinant to *E. coli* J53-1 chromosomal DNA. It was tested by PCR amplification.

Four oligonucleotides used for amplification of *tehA* and *tehB* genes were designed based on the published nucleotide sequence (Walter, *et al.*, 1991). They had been used to amplify *tehA* and *tehB* genes from *E. coli* J53-1, pUC8 and pDT1364. Amplification of *tehA*, *tehB* and *tehAB* from pDT1364 generated three DNA fragments of the expected sizes according to the known DNA sequence of *tehA* and *tehB* genes. No DNA fragments were generated from pUC8 DNA which had been used as a vector to construct pDT1364. These results indicate that the Te^r genes of pDT1364 did not originate from its vector pUC8. The three DNA fragments, *tehA*, *tehB* and *tehAB* were also amplified from *E. coli* J53-1which indicates that both genes *tehA* and *tehB* are not only present in *E. coli* J53-1 but also are arranged in the same way as they are in pDT1364, that is as neighbouring genes. These results suggest that the insert present in pDT1364 originated from the *E. coli* K-12 chromosome.

This result was further verified by localization of *tehA* and *tehB* genes on the Kohara miniset library which consists of 476 lambda phage clones covering the entire *E. coli* genome with ample overlapping. The Kohara miniset is available in an ordered array supplied either on six 96-well microtiter plates or on a single sheet of hybridization membrane, the *E. coli* Gene Mapping Membrane. The position of *tehA* and *tehB* genes were mapped at a position approximately between kb1508 and kb1510 (32.3 min coordinate) based on the hybridization of *tehA* and *tehB* genes with three consecutive and overlapping clones #270-272. To confirm this location, the three positive phage clones #270-272 and two other clones #170-171 were obtained from the Kohara miniset plates. Their DNAs were isolated, purified and subjected to dot-blot hybridization with radiolabeled *tehA* and *tehB* genes. As shown in FIG. 12, DNA of #270 and #271 hybridized to *tehA*; DNA of #271 and #272 hybridized to *tehB*; whereas no hybridization was observed between either of the probes and the two negative control clones. These results comfirmed the presence of *tehA* and *tehB* genes on *E. coli* K-12

chromosome. Further evidence was provided by the complete alignment of the restriction map of the 6.8kb insert on pDT1364 with that of the *E. coli* chromosome at a region from kb 1503 to 1510. Final proof was provided by the absence of *tehA* and *tehB* genes in the deletion mutant PK1439. This evidence allowed me to conclude that the 6.8kb insert of pDT1364 had originated from the *E. coli* K-12 chromosome. It is likely that the *E. coli* K-12 chromosomal DNA was present as a contaminant in the preparation of pHH1508a DNA which was used to clone the Te^r determinant from the plasmid.

All plasmid DNA used in Southern hybridization and cloning procedures was isolated by a large-scale preparation modified from the method of Birnboim and Doly (1979), purified by cesium chloride-ethidium broimide density gradient centrifugation. The purification depends on the difference between the buoyant density of the covalently closed circular (CCC) form of the plasmid molecules and those of linear fragments and open circular (OC) plasmid molecules. When ethidium bromide binds to DNA by intercalation between the bases, it causes the DNA double helix to unwind so that the lengths of the DNA molecules increase and their buoyant densities decrease. When ethidium bromide binds to CCC plasmids, the unwinding sequentially causes compensatory superhelical twists in the opposite direction which prevent further binding of ethidium bromide. The linear fragments and OC plasmid molecules are not constrained in the same way as CCC plasmids so that they bind more ethidium bromide and unwind more than that of CCC plasmids in the same condition. Bacterial chromosome, which is naturally circular, has been broken into linear fragments since cells are lysed during the alkaline extraction. Therefore CCC plasmid DNA has a greater buoyant density than chromosomal DNA and forms a band below that followed by the chromosomal DNA. In this way plasmid DNA is separated from bacterial chromosome and purified. However very large plasmids are very easily nicked and broken by shear force when the cells are lysed. They are particularly difficult to keep in the CCC form

during isolation and purification. So that their DNA can not be separated from chromosomal DNA and thus contamination occurs. Plasmid pHH1508a has large molecular weights with an estimated size of 208 kb (Yan, M.Sc. thesis, 1986). Its large size makes it very difficult to purify and thus the DNA is usually contaminated by chromosomal DNA. The fact that the pDT1364 Te^r determinant hybridized with a fragment which does not belong to pHH1508a in Southern hybridization confirms the presence of contaminating DNA in the pHH1508a preparation. Hybridization of *E. coli* J53-1 chromosomal DNA to pDT1364 Te^r determinant provides evidence that the contamination might be from *E. coli* K-12 chromosome. However, it is not clear why the chromosomal Te^r determinant was cloned in preference to the Te^r determinant in plasmid pHH1508a.

As shown in this study, the *tehAB* genes in pDT1364 have originated from *E. coli* K-12 chromosome instead of the IncHII plasmid pHH1508a. They were found to be widely conserved in *E. coli* laboratory strains by PCR. Ten *E. coli* K-12 strains as well as *E. coli* B/R, C and W contain the *tehAB* genes. In order to define the prevalence of *tehAB* genes in natural isolates of *E. coli*, the 72 standard reference strains (the ECOR Collection) were tested for the possible presence of *tehA* and *tehB* genes by dot blot hybridization. The *tehA* gene was found to be present in all the ECOR strains, except for ECOR 17, whereas the *tehB* gene was lacking in ECOR 9, ECOR 12, ECOR 17 and ECOR 30. To confirm these results, PCR reactions were carried out using primers for *tehA*, *tehB* and 23S rRNA genes. The results (FIG. 18) demonstrated that only ECOR 17 contained neither *tehA* nor *tehB* whereas ECOR 9, ECOR 12 and ECOR 30 contained both genes. This indicates that PCR, which can amplify even from one single DNA molecule, is much more sensitive than dot blot hybridization. The absence of hybridization of the *tehB* gene to ECOR 9, ECOR 12, ECOR 17 and ECOR 30 is probably due to the small amount of DNA loaded onto the filters used for dot blots.

Twelve other bacterial species including *Salmonella typhimurium* LT2, *Shigella sonnei*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Arizona* spp., *Citrobacter*, *Proteus vulgaris*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, *Campylobacter jejuni*, *Corynebacterium diphtheriae* and *Staphylococcus aureus* were also tested for the presence of *tehA* and *tehB* genes by PCR and dot blot hybridization. None of them contained these genes. Thus the *tehAB* genes are highly specific for *E. coli*.

Although the *tehAB* genes result in a high level of Te^r with a MIC of K_2TeO_3 128μg/ml in pDT1364 using pUC8 as a vector, they usually do not specify Te^r when they are expressed in *E. coli* chromosome. Most of the *E. coli* strains had MICs of 2 to 8μg/ml. Only one strain, *E. coli* BB4 had a very high MIC over 256μg/ml. A few strains including JC1569, JM105 and DH5α were very susceptible to K_2TeO_3 and had very low MICs at 0.4, 0.1 and 0.64μg/ml. The different levels of Te^r are probably related to the level of expression of the operon. When the genes are present in pDT1364, they are present in high copy number, whereas when they are expressed in the *E. coli* chromosome, they are usually present only in a single copy. The high level of Te^r encoded by BB4 is possibly due to the presence of multiple copies of *tehAB* genes in *E. coli* BB4 chromosome or of a mutation in the promotor in front of *tehA* which upregulates the expression of the *tehA* and *tehB* gene. The copy number of the *tehAB* genes in this strain should be tested or other possible mutation(s) need to be investigated in the future. The low MICs encoded by the strains of JC1569, JM105 and DH5α are probably due to presence of other mutation(s) in their chromosomes which have been found to cause reduced MIC of Te^r. These mutations are involved in some genes in the cysteine biosynthesis pathway or in the electron transport pathway (Turner, *et al.*, unpublished data).

The *tehAB* genes are found to be widely conserved in the species of *E. coli*. Only two *E. coli* strains, PK1439 and ECOR 17 were found to be devoid of *tehAB* operon by PCR or by hybridization. The large deletion mutant PK1439, containing a 140-kb deletion within the terminus region, specified an MIC (0.2μg/ml) about 20 times lower than most other *E. coli* strains. However, the introduction of pDT1364 into the strain did not restore the Te^r and the MIC retained at 0.2μg/ml. Thus it is likely that other mutations are involved. These mutations need to be investigated in future work. In spite of the absense of the *tehAB* genes in ECOR 17, the strain still specified an MIC of 2μg/ml which is very similar to the MICs of most *E. coli* strains carrying chromosomal *tehAB* genes. However, Te^r was fully complemented by pDT1364 in ECOR 17. Therefore it can be concluded that: (1) the *tehAB* genes do not result in Te^r unless they are expressed at high levels; (2) other genes, as yet unidentified are required for expression of Te^r by the *tehAB* genes. The unidentified genes and the *tehAB* genes were not found to have any effects on Te^r encoded by the IncHI2 and IncPα Te^r determinants, as the MICs by these determinants in either PK1439 or ECOR 17 were shown to be the same as those in the wild-type strains.

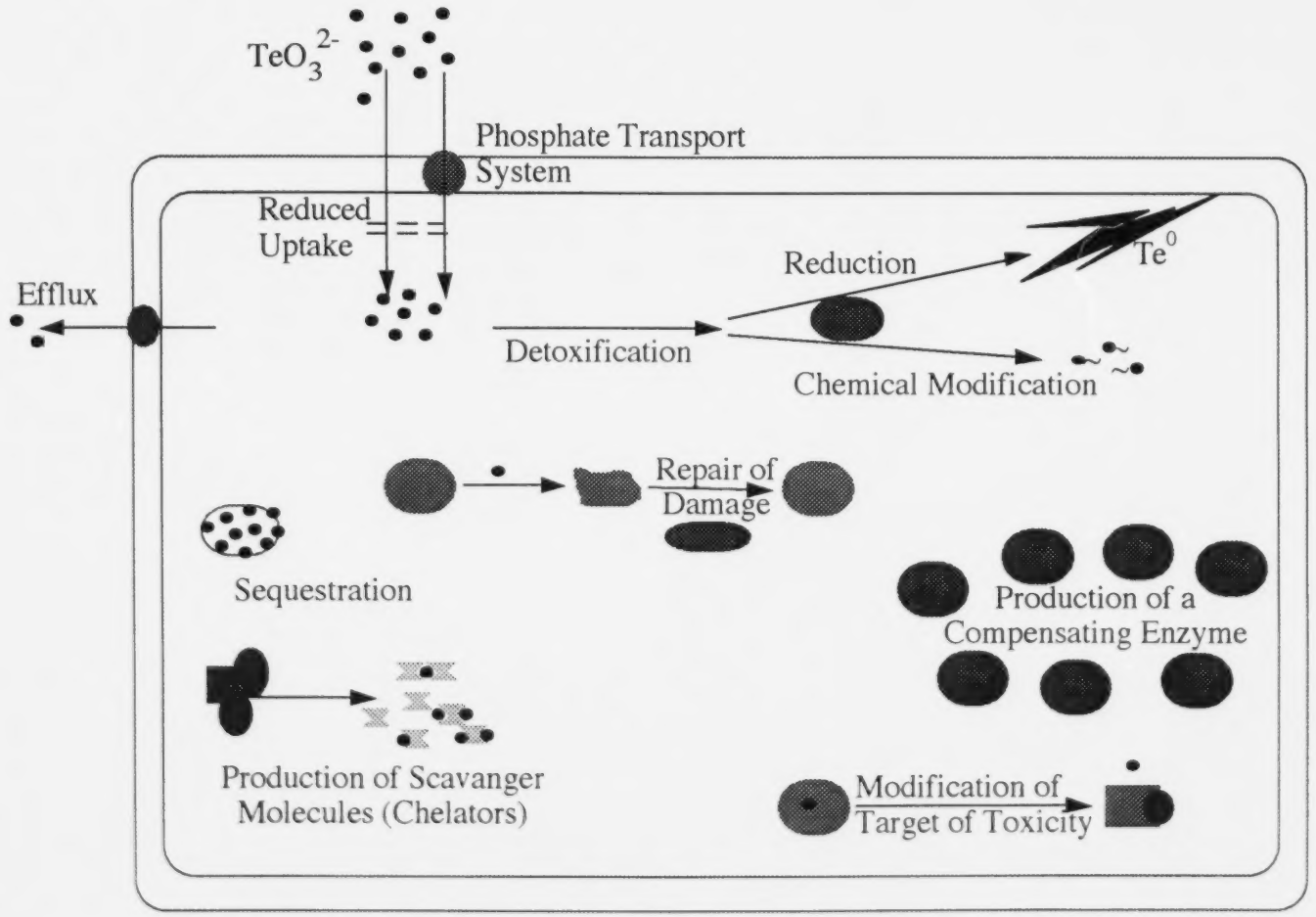
Now that the identification of the origin of *tehAB* has been resolved, it is possible with greater confidence to investigate the mechanism by which these genes mediate resistance to potassium tellurite. Figure 10. shows a cartoon representing possible methods in which gram-negative bacteria could employ to deal with the toxic oxyanion tellurite. The mechanisms of reduced uptake (efflux) and sequestration have already been eliminated (Turner, *et al.*, in preparation I).

The conservation of the *tehAB* genes in the majority of *E. coli* strains may indicate that the genes have important functions. However the genes do not appear to be essential for the strains. The *tehAB* genes which specify Te^r when present in high copy number

have been shown to be located in the terminus region. Moir *et al.*(1992) have suggested that the terminus region encodes apparently non-essential proteins. Nevertheless other possible functions of the *tehAB* genes need to be investigated.

FIG 20. Possible mechanisms of tellurite resistance in gram-negative bacteria.

(This picture was kindly supplied by Dr. R.J. Turner)


TeO_3^{2-}
Phosphate Transport System
Reduced Uptake
Efflux
Reduction
Te^0
Detoxification
Chemical Modification
Repair of Damage
Sequestration
Production of a Compensating Enzyme
Production of Scavanger Molecules (Chelators)
Modification of Target of Toxicity

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Appendix I. Media and Solutions

Media used for isolation of bacteriophage DNA were made up as follows:

10 mM $MgSO_4$-LB plates, per liter

Tryptone	10 g
Yeast extract	5 g
NaCl	5 g
Agar	15 g
1M $MgSO_4$	10 ml

LB-top agar, per liter

Tryptone	10 g
Yeast extract	5 g
NaCl	5 g
Agar	7 g

Before use, the agar was melt by heating in a microwave oven and cooled to 45°C to 50°C.

SM (suspension medium), per liter

NaCl	5.8 g
$MgSO_4 \cdot 7H_2O$	2 g
1M Tris·(pH 7.5)	50 ml
2% gelatin solution	5 ml

This buffer was used for storage and dilution of bacteriophage lambda stocks.

2xYT/MT medium, per liter

Tryptone	16 g	
Yeast extract	10 g	
	NaCl	5 g
2M Tris·(pH7.5)	5 ml	
1M $MgCl_2$	2 ml	

All the media used were autoclaved at 15 Lb/in^2 for 15 min. For preparing plates, agar was cooled to 50^{o}C after autoclave, poured into sterile disposable petri dishes and left to solidify. Then the plates were dried by leaving them at room temperature for 2 days or by leaving them with the lids off for 30 min to 1 hour in a laminar flow hood. Antibiotics, potassium tellurite and some nutritional supplements were added only after the medium had cooled to 50^{o}C or below.

The stock solution of gel electrophoresis buffer was prepared as follow:

50x TAE (Tris-acetate / EDTA), per liter

Tris-base	242.24 g
Glacial acetic acid	57.1 ml
0.5M EDTA (pH 8.0)	100 ml

Appendix II Restriction Endonucleases and Digestion Conditions[a]

Enzyme	Buffer[b]
*Bam*HI	B
*Bgl*I	H
*Bgl*II	M
*Eco*RI	H
*Eco*RV	B
*Hin*dIII	B
*Kpn*I	L
*Nde*I	H
*Pst*I	H
*Pvu*II	M
*Sma*I	A
*Xba*I	H
*Xho*I	H

[a] The temperature of incubation was at 37°C

[b] Boehringer Mannheim SuRE/Cut buffers